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Our long term goal of this proposal was to study the role of elk-1 gene in the progression of breast cancer. Our studies on the expression of elk-1 in sporadic breast cancer suggests that changes in the levels of expression of elk-1 protein can lead to breast cancer. Elk-1 proteins were found to be involved in the estrogen induced signal transduction pathway and are targets for c-src kinase. Constitutive expression of elk-1 and Δelk-1 proteins in rat fibroblasts and human breast cancer cells induces apoptosis. Similarly, high level expression of BRCA1 protein in mouse fibroblasts and human breast cancer cells induces apoptosis on serum withdrawal or calcium ionophore treatment. These results suggest that elk-1 and BRCA1 genes play a critical role in the regulation of apoptosis of human breast cancer cells. We have found the BRCA1 protein to associate with E2F transcriptional factors, Cyclins, Cyclin dependent kinases, CBP and p53 protein suggesting a function for these proteins in cell cycle regulation, tumor suppression, transcription activation, etc. Breast cancers have a reduced ability to undergo cell death. Treatments aimed at increasing the apoptotic threshold by BRCA1 and elk-1 gene therapy may have the potential to prevent the progression of these cancers.

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INTRODUCTION

The ets-oncogene superfamily contains a large number of genes sharing a conserved region of 85 amino acid called the "ETS domain" and a majority of the ets-oncogene superfamily members, which we and others have previously isolated, cloned and characterized are involved in leukemias, lymphomas and solid tumors (1-8). One of the members elk-1 (4) was shown to form a SRF (serum response factor) dependent ternary complex with the SRE (serum response element) similar to P62TCF (ternary complex factor) (9). Elk-1 was previously shown to be involved in the growth factor mediated signal transduction pathway involving activation of MAP kinase (mitogen activated protein kinase), leading to the transcriptional activation of c-fos proto oncogene both of which are activated in tumor cells. Since the fos regulatory function of elk-1 is dependent on the presence of a growth factor regulated transcriptional activation domain whose activity is dependent on phosphorylation by MAP kinase (10-13) and JNK kinases (Jun amino terminal kinase) (14) *in vivo* both of which are activated in tumor cells, we speculated that elk-1 proteins may be obligatory intermediates in the estrogen and growth factor mediated signal transduction pathway leading to the progression of breast cancer. We have tested this hypothesis by studying the levels of expression of elk-1 protein in breast tumor samples as well as in several breast cancer derived tumor cell lines and compared expression with histologically normal breast tumor samples by western blot analysis using the elk-1 polyclonal antibody to correlate expression

with stages of the disease. We observed high levels of expression of elk-1 protein (~3-8 fold higher) in samples derived from invasive breast cancer than normal breast. These results suggested that elk-1 proteins may be involved in the progression of breast cancer.

Breast cancers have been described in patients overtime to change from an estrogen dependent in initial stages of the disease to a hormonally independent tumor. We therefore studied the expression of elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle after the addition of E2 by RT-PCR analysis. We found a rapid induction of elk-1 and Δelk-1 RNA following growth stimulation by E2 in MCF-7 cells, indicating the involvement of elk-1 in the E2 induced signal transduction pathway in human breast cancer cells. Furthermore we observed no significant difference in the expression of elk-1 protein on immunoprecipitation analysis of ^{35}S methionine labeled MCF-7 cells exposed to estrogen for different time intervals. One explanation for this could be that MCF-7 cells have been maintained *in vitro* in tissue culture for a long time and hence may have drifted far from their origin *in vivo*, which could explain the results found in breast tumor samples. As suggested by one of the reviewers, MCF-7 cells are E2 responsive and non-metastatic, whereas MDA-MB-231 cells or CAL-51 cells are estrogen independent, highly invasive and metastatic. We therefore plan to compare the expression of elk-1 in these cell lines which would indicate the role of elk-1 in early i.e., E2 dependent and late (invasive) breast cancer cell growth.

Since steroid hormone estrogen and peptide growth factors stimulate cellular proliferation of human breast cancer cells by inducing tyrosine phosphorylation of src family protein kinases and expression of c-fos proto oncogene, we subjected purified recombinant elk-1 protein to *in vitro* kinase assay using src kinase. Our results suggests elk-1 protein to be a target for c-src kinase.

We have previously investigated and found that high level expression of elk-1 or ∆elk-1 protein transforms mouse fibroblasts *in vitro* and induces tumors in nude mice indicating that elk-1 and ∆elk-1 proteins are potentially tumorigenic. These results correlated well with the results obtained using breast tumor samples. One should bear in mind that breast cells are epithelial in origin unlike mouse/rat fibroblasts which are mesenchymal.

Recently a familial breast and ovarian cancer susceptibility gene BRCA1 was identified (15) and shown to be either lost or mutated in families with breast and ovarian cancer (16-20). Earlier, results on the expression of BRCA1 mRNA in sporadic breast cancer indicated decrease in the level of BRCA1 mRNA levels during the transition from carcinoma in situ to invasive cancer (21). But recent results of us and others have indicated increase in the expression of BRCA1 in tumor compared to normal samples, similar to elk-1 protein and BRCA1 expression has been correlated with the proliferation state of the cell (27, 28). At this juncture, we were tempted to look for any relation between BRCA1 expression and elk-1

expression or vice versa. So, in order to understand the role of BRCA1 in cell transformation, we obtained stable NIH3T3 cell lines expressing BRCA1 antisense RNA. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental transfectants (25, reprint enclosed). These results provide direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene. These results suggested that inhibition of expression of BRCA1 protein is sufficient to achieve transformation and BRCA1 functions as a growth regulator in normal cells. Similar results were obtained by others using BRCA1 antisense oligonucleotides in MCF-7 cells (21). Previously, we have performed the SRE TK CAT (Serum Response Element, Thymidine Kinase, Chloramphenicol acetyl transference reporter vector) functional assay for checking the levels of elk-1 protein in cells that have been transfected with antisense RNA to BRCA1 ($BRCA^{AS}$) that have been developed by us. We observed high levels of CAT activity which made us to speculate that BRCA1 could be a regulator of elk-1 gene. But we know that there are many ets-1 related proteins which would also respond to this reporter like SAP-1, SAP-2, Fli-1, etc. and hence, these results need to be confirmed by western blot analysis.

In order to study whether any relationship exists between BRCA1 and elk-1 we have developed mouse fibroblasts cell lines and human breast cancer cell lines expressing BRCA1 (26, see enclosed reprint). We have found that serum deprivation or calcium ionophore

treatment of BRCA1^S transfectants resulted in programmed cell death (26, see enclosed reprint). These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis of human breast cancer cells. We have examined the expression of elk-1 protein in the MCF-7 BRCA1^S cells by western blot analysis and have observed higher levels of expression of elk-1 compared to vector transfected MCF-7 cells. These results suggested that elk-1 could be a target for BRCA1. In order to test the hypothesis whether elk-1 lies downstream of BRCA1 in the apoptotic pathway and to investigate the role of elk-1 in apoptosis of breast cancer cells, we have developed rat fibroblast cell lines and human breast cancer cell lines expressing elk-1 proteins. We have found that constitutive expression of elk-1 and Δelk-1 proteins induce apoptosis when cells are treated with calcium ionophore. These results suggest that elk-1 proteins may be downstream targets and inducers of apoptosis in human breast cancer cells. Recent evidence suggests that hormone dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. It may be possible that inability to undergo apoptosis in these cancers may be due to the decreased levels of functional elk-1 and BRCA1 proteins. These experiments will give us a clue as to the possible regulation of elk-1 function in breast cancer. Future experiments will be directed towards studying the role of elk-1 in apoptosis/growth and tumor suppression. It may be possible that treatments that are aimed at increasing the apoptotic threshold by elk-1/BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

BODY

Task 1 COMPLETED

As mentioned earlier, in an attempt to link elk-1 gene to breast cancer, which forms the foundation for the work proposed in this proposal and represents Task 1 of this grant proposal, we have isolated total RNA from ~20 different sporadic cases of breast cancer and various breast cancer derived cell lines that are in different stages of the disease. Of these samples some are sets of paired samples which contain both normal breast and breast cancer from the same patient, the rest are unpaired. All these samples have been evaluated histologically by frozen sectioning and H & E (Hematoxylin and Eosin) staining as normal or malignant. We subjected these total RNA (~20 μg) samples to Northern blot analysis as described previously (4). The Northern blot was hybridized to elk-1 specific probe. Equal amounts of RNA was loaded in all the lanes. The signal obtained on Northern blot was very weak and the results were inconclusive so we resorted to alternative strategies like reverse transcriptase PCR (RT-PCR) of total RNA samples using elk-1 ATG and termination primer which can distinguish between elk-1 and Δelk-1 transcripts. We used human β-actin primer (Clontech) as internal controls. Once again, we could not detect clear elk-1 and Δelk-1 bands by RT-PCR.

Since the elk-1 mRNA levels doesn't necessarily reflect differences in the level of elk-1 protein, we studied the expression of elk-1 at the protein level by Western blot analysis using the elk-1 polyclonal antibody available with us. It is because of these negative results that we had to deviate to alternate strategies like Western blot for studying the expression of elk-1 protein. We observed elevated levels of expression of elk-1 protein ~3-8 fold higher in samples derived from invasive breast cancer than in normal breast. Thus, our results indicating alterations in the expression of elk-1 during the progress from normal to invasive breast cancer has strengthened the objectives of this proposal. **WITH THESE RESULTS WE HAVE COMPLETED TASK 1 OF THIS PROPOSAL.**

Task 2 **IN PROGRESS**

Task 2 (a) **COMPLETED**

To test the hypothesis whether elk-1 protein are intermediates in the E2 mediated proliferation of breast cancer cells, we have studied the expression of elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle by the addition of E2 for 60 minutes as described in the proposal followed by RNA isolation. Total RNA from both control untreated and E2 stimulated were subjected to double stranded cDNA synthesis, RT-PCR analysis and Southern blot using elk-

1 ATG and internal primers which can distinguish between the two alternately spliced forms elk-1 and ▵elk-1. The RT-PCR analysis was also repeated using human β-actin, 5' and 3' primers as internal controls. We find rapid induction of elk-1 and ▵elk-1 RNA following growth stimulation by E2 of MCF-7 cells. These results indicate involvement of the elk-1 protein in the E2 induced signal transduction pathway in human breast cancer cells. Similarly, we have also examined the expression of elk-1 protein in MCF-7 cells that have been stimulated to proliferate in the presence of E2 for different periods and have not observed any increase in the levels of elk-1 protein. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (a).**

Task 2 (b) **COMPLETED**

We have studied the autonomous and SRF dependent transcriptional activation of elk-1 and ▵elk-1 proteins in MCF-7 cells that have been stimulated with E2 and growth factors. We have observed no difference in the transcriptional activation of elk-1 proteins in presence of E2 and growth factors. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (b).**

Task 2 (c) **COMPLETED**

We have subjected purified recombinant elk-1 protein to *in vitro* kinase assay, using purified c-src kinase. The elk-1 protein was phosphorylated by c-src kinase, indicating that elk-1 could be a physiological target of c-src kinase.

Task 2 (d) **IN PROGRESS**

We have grown and collected the cell pellets of MCF-7 cells (E2 responsive and non-metastatic and CAL-51 cells (E2 independent, highly invasive and metastatic). We plan to study the expression of elk-1 proteins by western blot analysis to determine the role of elk-1 in early versus late breast cancer cell growth.

Task 2 (e) **COMPLETED**

In an attempt to identify proteins that might interact with elk-1 proteins, we have used an alternate methodology other than the yeast match maker two-hybrid system. Since elk-1 is a physiological substrate for MAP kinase and has multiple MAPK phosphorylation sites, we purified and labeled the protein *in vitro* using MAP kinase and used it to screen a human breast tumor GT11 expression cDNA library. We have obtained several clones in the primary screening and are in the process of confirming and characterizing these cDNA clones.

Task (f) **COMPLETED**

Since we found elevated levels of expression of elk-1 in breast tumor samples, we wanted to investigate whether high level expression of elk-1 or ΔElk-1 proteins could lead to cell transformation. We have recently obtained stable BALB and Rat-1 cells expressing high levels of elk-1 proteins based on immunoprecipitation, western analysis and functional assay. In these cells we have observed stimulation of transactivation of the fos promoter CAT reporter or SRE-TK CAT reporter plasmid unlike the control Rat-1 cells. We have used this as a functional assay for testing the presence of elk-1 proteins. We find ~20 fold increase in reporter CAT activity after transfection of SRETKCAT reporter into Rat-1 elk-1 cells when compared to parental Rat-1 cells. The elk-1 and ΔElk-1 expressing Rat-1 cells showed anchorage independent growth, formed colonies in soft agar induced tumors in nude mice, unlike the control cells. These results suggests that elk-1 gene has transforming properties at least in mesenchymal cells. **WITH THIS WE HAVE COMPLETED TASK 2 (f).**

Task 2 (g) **COMPLETED**

As mentioned previously, BRCA1 a familial breast and ovarian cancer susceptibility gene, was cloned and shown to be lost or mutated in families with breast and ovarian cancer resulting in loss of function of the BRCA1 protein. Since the reported BRCA1 cDNA

sequence is a composite of several partial cDNA sequences, we obtained a novel full length alternately spliced BRCA1 cDNA clone (33). Recently we have also cloned the variant BRCA2 cDNA (38) and have studied its function (39). To test the hypothesis whether BRCA1 functions as a tumor suppressor gene, we have used antisense RNA methodology (22, 23) and cloned the full length antisense BRCA1a cDNA into pcDNA3 expression vector (25). We have previously used this method to inhibit Ewing's sarcoma in mice (23, 24). We have transfected NIH3T3 cells with pcDNA expression vector containing antisense BRCA1 cDNA and obtained stable neo-resistant cell lines expressing antisense RNA to BRCA1. These cell lines showed ~5 fold decrease in the expression of endogenous BRCA1 protein by western blot analysis (25). The $BRCA1^{AS}$ cells showed accelerated growth rate, anchorage independent growth, tumorigenicity in nude mice unlike the parental cells (25). Transfection of SRE TK CAT reporter plasmid into $BRCA1^{AS}$ cells showed high levels of CAT activity unlike the parental NIH3T3 cells. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor. This high level of SRE TK CAT activity made us to speculate that BRCA1 could be a regulator of elk-1 gene. But we know that there are many ets-1 related proteins which respond to this reporter like SAP1, SAP2, Fli-1, etc. and hence these results have to be confirmed by western blot analysis. **WITH THESE RESULTS WE HAVE COMPLETED TASK (g).**

Task 2 (h) **IN PROGRESS**

We plan to examine expression of elk-1 proteins in mouse fibroblast cells that have been transfected with antisense BRCA1 by western blot analysis

Task 2 (i) & (j) **COMPLETED**

Since our preliminary results suggested a link between elk-1 and BRCA1 proteins, we studied the subcellular localization, phosphorylation and protein-protein interactions of BRCA1 proteins. Recently, we have characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine (33, reprint enclosed). Immunofluorescence and western blotting analysis indicated cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins (33, reprint enclosed). Recently, we have also shown the amino-terminal region of BRCA1a (BNT) but not BRCA1b can function as a transcriptional activator when fused to GAL4 DNA binding domain (34, reprint enclosed). Thus, BRCA1/1a proteins contain two autonomous transcriptional activation domains, one at the amino-terminal region (BNT) and the other at the carboxy-terminal region (BCT) (34). In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1 (33, reprint enclosed). Western blot analysis of BIP indicated association with

E2F, cyclins, and CDK's and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factors E2F-1, E2F-4, cyclins A, B1, D1 and cyclin dependent kinases cdc2 and cdk2 suggesting a role for BRCA1 proteins in cell cycle regulation (33, reprint enclosed).

Recently, we have also found BRCA1a/1b proteins to interact both *in vitro* and *in vivo* with the carboxy-terminal domain of transcription factor CBP (35, reprint enclosed) as demonstrated by mammalian two-hybrid assays, co-immunoprecipitation/western blot studies, GST binding assays and histone acetyltransferase (HAT) assays of BRCA1 immunoprecipitates from human breast cancer cells (35, reprint enclosed).

Our recent results suggest BRCA1a and BRCA1b proteins function as co-activators of p53 tumor suppressor protein similar to BRCA1 (36, preprint enclosed). This study demonstrates for the first time the presence of a second p53 interaction domain in the carboxy-terminal BRCT domain of BRCA1 which is sufficient for activation of p53 dependent transactivation of the $p21^{WAF1/CIP1}$ promoter (36). The BRCA1a/1b proteins interact with p53 both *in vitro* and *in vivo* The BRCT domain binds to the central domain of p53 which is required for sequence specific DNA binding (36). This BRCT domain also binds *in vitro* to CBP. These results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation (36).

In order to study whether any relationship exists between BRCA1 and elk-1 we have developed mouse fibroblasts cell lines and human breast cancer cell lines expressing BRCA1 (26, see enclosed reprint). We have found that serum deprivation or calcium ionophore treatment of $BRCA1^S$ transfectants resulted in programmed cell death (26, see enclosed reprint). These results suggested that elk-1 could be a potential target for BRCA1. **WITH THIS WE HAVE COMPLETED TASK 2 (i) (j).**

Task 2 (k)

Previously we have observed high levels of expression of elk-1 protein in MCF-7 cells that have been transfected with BRCA1 compared to vector transfected MCF-7 cells by western blot analysis using elk-1 polyclonal antibody. These results suggested that BRCA1 gene may regulate the expression of elk-1 or elk-1 could be a downstream target for BRCA1 in the apoptotic pathway. Since BRCA1 is an inducer of apoptosis we speculated whether elk-1 could also be involved in apoptosis. Recently, mitogen activated/extracellular response kinase kinase kinase (MEKK), a serine-threonine kinase that regulates phosphorylation and activation of mitogen-activated protein kinases (MAPK) was shown to induce cell death (29). Inducible expression of activated MEKK (which induces apoptosis) stimulated the

transactivation of c-Myc and elk-1 (29). c-Myc and c-jun transcription factors which are regulated by MAPK phosphorylation also induce apoptosis (30). Since elk-1 protein regulates c-Fos oncogene and is a target for MAPK and JNK both of which are activated by MEKK, we speculated whether it could play a similar role in inducing an apoptotic response. To test this hypothesis, we have transfected Rat-1 fibroblasts with pcDNA expression vector or pcDNA expression vectors containing human Elk-1 and ΔElk-1 cDNA and obtained stable G418 resistant cell lines expressing Elk-1 and ΔElk-1 proteins as described previously (26).

Analysis of expression of Elk-1 and ΔElk-1 proteins in Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants

We analyzed the expression of Elk-1 and ΔElk-1 proteins in Elk-1 and ΔElk-1 transfectants by immunoperoxidase staining (37, reprint enclosed), indirect immunofluorescence analysis (37) and western blot analysis (37) using Elk-1 polyclonal antibody as described previously (26 and 33). The nuclear and cytoplasmic staining was brighter and stronger in both Elk-1 and ΔElk-1 transfected cells compared to parental Rat-1 fibroblast cells suggesting higher level of expression of Elk-1 and ΔElk-1 proteins than Rat-1 cells. Western blot analysis revealed a significant increase in the levels of expression of Elk-1 and ΔElk-1 proteins when compared to the parental Rat-1 cells (37).

Constitutive Elk-1 and ΔElk-1 expression induces cell death in Rat-1 cells after calcium ionophore treatment

Previously, we have shown calcium ionophore A23187 to induce apoptosis in BRCA1a transfected NIH3T3 and MCF-7 cells (26). This led us to examine apoptosis in Rat-1 Elk-1 and Rat-1 ΔElk-1 transfectants after A23187 treatment. Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 were treated with calcium ionophore A23187 for 48 hours and the cell cycle distribution was determined by flow cytometry with propidium iodine staining method (31). Histogram of the DNA content and the percentage of cells in G1, S, and G2 plus M phase of the cell cycle were evaluated using EPICS profile analyzer. The Elk-1 and ΔElk-1 transfected cells showed accelerated rates of apoptosis (Ap value 49% for Rat-1 Elk-1 and 42% for Rat-1 ΔElk-1 cells) in the presence of calcium ionophore (37) whereas the control Rat-1 fibroblasts cells showed lower levels of apoptosis under identical conditions (Ap value 16%).

Apoptosis in the Rat-1 Elk-1 and Rat-1 ΔElk-1 cells confirmed by cell viability staining

Measurement of apoptosis through the sub G1 peak in the DNA histogram gives no distribution between viable and dead cells since all the cells are fixed. We, therefore, studied the viability of Rat-1 cells, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells cultured in the presence of calcium ionophore by crystal violet staining. Cell viability staining showed that most of the

Elk-1 and ΔElk-1 cells treated with calcium ionophore A23187 were dead whereas most of the control Rat-1 cells survived (37). These results suggest that constitutive expression of Elk-1 and ΔElk-1 proteins induce death in Rat-1 fibroblast cells.

Apoptosis in the Rat-1 Elk-1 and Rat-1 ΔElk-1 cells confirmed by chromatin condensation

Elk-1 and ΔElk-1 transfected Rat-1 cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hochest 33258 (32). Majority of the nuclei of Rat-1 Elk-1 and Rat-1 ΔElk-1 cells showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis. Whereas the parental Rat-1 cells did not show any significant change in the staining pattern (37).

Apoptosis in the Elk-1 and ΔElk-1 transfectants confirmed by DNA fragmentation

The induction of apoptosis in the Elk-1 and ΔElk-1 transfectants upon treatment with calcium ionophore was further confirmed by analysis of DNA fragmentation. The DNA of Elk-1 and ΔElk-1 cells treated with calcium ionophore was broken into oligo nucleosomal DNA ladder typical of apoptosis whereas the parental Rat-1 cells had no significant DNA

degradation (37). These results suggested that calcium ionophore induces apoptosis more readily in Rat-1 Elk-1 and Rat-1 ΔElk-1 cells than Rat-1 cells. All the above results shown for a single clone of Rat-1 Elk-1 or Rat-1 ΔElk-1 cells have been reproducibly obtained with several other independent clonal isolates of Rat-1-Elk-1 and Rat-1-ΔElk-1 cells. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (k).**

Task 3

Task 3(a) **COMPLETED**

In an attempt to study the role of Elk-1 gene in the regulation of apoptosis of human breast cancer cells, we have transfected MCF-7 cells with pcDNA expression vector or pcDNA expression vector containing human Elk-1 and ΔElk-1 cDNA's and obtained stable G418 resistant cell lines expressing Elk-1 and ΔElk-1 proteins.

Morphology of MCF-7 Elk-1 and MCF-7 ΔElk-1 transfectants

The morphology of the Elk-1 transfectants were different than that of the parental Rat-1 fibroblast cells. The Elk-1 transfectants were slow growing and appeared to be flatter and larger compared to the parental MCF-7 cells. The morphology of the ΔElk-1 transfectants

were similar to that of the parental MCF-7 cells (37). These MCF-7 Elk-1 and MCF-7 ΔElk-1 cell lines were analyzed for Elk-1 and ΔElk-1 protein expression by immunoperoxidase staining and western blot analysis and found to express Elk-1 and ΔElk-1 proteins.

Constitutive expression of Elk-1 and ΔElk-1 proteins induces apoptosis in MCF-7 cells treated with calcium ionophore

Apoptosis in the MCF-7 Elk-1/ΔElk-1 transfectants were analyzed after treatment with calcium ionophore A23187. MCF-7 and MCF-7 Elk-1/ΔElk-1 cells were treated with calcium ionophore A23187 for 24 hours and the cell cycle distribution was analyzed by flow cytometry with propidium iodide staining. The Elk-1 transfected MCF-7 cells showed accelerated rates of apoptosis (Ap value 72%) in the presence of calcium ionophore (37), unlike the parental MCF-7 cells which showed very low levels of apoptosis (Ap value 4%). The MCF-7 ΔElk-1 cells showed low levels of apoptosis in presence of calcium ionophore (Ap value 14%). The viability of MCF-7 cells, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells cultured in the presence of calcium ionophore were tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 Elk-1 cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived. These results suggest that Elk-1 induces death in MCF-7 cells. The induction of apoptosis in the MCF-7 Elk-1 and MCF-7 ΔElk-1 transfectants was further confirmed by DNA fragmentation

analysis upon treatment with calcium ionophore. The DNA of MCF-7 Elk-1 cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (37). The DNA fragmentation observed in MCF-7 ΔElk-1 cells was much less. These results suggest that overexpression of Elk-1 in breast cancer cells accelerates apoptosis. We plan to identify the role of DNA binding, transcriptional activation and protein protein interactions on the apoptosis inducing function of elk-1 proteins in breast cancer cells.

Task 3 (b) (c) (d) **IN PROGRESS**

We have introduced Elk-1 into E2 independent, highly invasive and metastatic breast cancer cell line CAL-51. We plan to study the effect on apoptosis and growth/tumor suppression. We also plan to study whether DNA binding, transcriptional activation and protein-protein interactions play a role in the apoptosis inducing, growth/tumor suppressor function of Elk-1 proteins. We have not been able to complete all the proposed work in the grant proposal due to insufficient funds available in the funded grant proposal. In fact, I had written a letter asking for additional funds for a year so that I could finish the work proposed but, unfortunately, this request was turned down by the U.S. Army Medical Research and Materiel Command.

CONCLUSION

Our studies are designed to investigate the role of elk-1 in the progression of breast cancer. In summary, our results suggest that BRCA1, which is a tumor suppressor and elk-1 gene product function as inducers of apoptosis in breast cancer cells. This study demonstrates for the first time a role for elk-1 proteins in mediating apoptosis.

Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. Our results suggest that lack or decreased levels of expression of functional BRCA1 or elk-1 gene products in breast cancer may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 or elk-1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1/elk-1 downstream signals involved in apoptosis for the treatment of breast cancers. Results from this work would be utilized in the future for early detection, diagnosis and also treatment of breast cancer.

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MEETING ABSTRACTS

1. Shao, N., Chai, Y.L., Reddy, E.S.P. and Rao, V.N. "BRCA1 induces apoptosis of human breast cancer cells." Presented at the International Meeting on programmed cell death at Shanghai, China, September, 1996.

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The EWS-ATF-1 gene involved in malignant melanoma of soft parts with t(12;22) chromosome translocation, encodes a constitutive transcriptional activator

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Molecular characterization of malignant melanoma of soft parts or soft tissue clear cell sarcoma which shares t(12;22) chromosome translocation revealed fusion of EWS with a transcriptional factor gene ATF-1. The EWS gene, which encodes an RNA binding protein, was also shown to be involved in Ewing sarcoma, related primitive neuroectodermal tumors and desmoplastic small round cell tumors. In order to understand the functional role of EWS-ATF-1 chimeric protein in human solid tumors, we have cloned the aberrant human ATF-1 (EWS-ATF-1) cDNA and studied its DNA binding, transcriptional activation properties and compared with normal ATF-1 protein. Our results demonstrate that EWS-ATF-1 binds weakly to DNA *in vitro* but functions as an efficient constitutive transcriptional activator unlike the normal ATF-1 which needs to be induced with cAMP. Deletion analysis revealed that EWS-fusion domain functions as a regulatory domain for the transcriptional activation properties of EWS-ATF-1 chimeric protein. Deletion of leucine zipper domain results in a loss of transcriptional activation of EWS-ATF-1 chimeric protein suggesting that protein-protein interaction play a role in the transcriptional activation properties of EWS-ATF-1. We demonstrate that EWS-fusion domain negatively regulates the DNA binding activity of EWS-ATF-1 chimeric protein. Therefore replacement of part of the amino-terminal kinase regulatory domain of ATF-1 protein with EWS regulatory domain results in an altered DNA binding, protein-protein interactions and transcriptional activation properties of EWS-ATF-1 causing deregulated gene expression which may be responsible for the genesis of t(12;22) chromosome translocation-bearing human solid tumors. Targeting the transcriptional cofactors (CBP, etc) by EWS-fusion proteins could be one of the mechanisms of activation of EWS-fusion proteins in human neoplasia.

Keywords: malignant melanoma; EWS; transactivation; ATF-1; chromosome translocation

Introduction

Recurrent chromosomal translocations have been described for hematological neoplasias (Mitelman, 1991). Molecular characterization of the majority of these translocations revealed fusion of genes encoding transcriptional factors/DNA binding proteins suggesting deregulation of gene expression may be responsible for these neoplasia (Mitelman, 1991; Rabbitts, 1994; Cleary, 1991). Analysis of recurrent chromosomal translocations found in the human solid tumors also revealed such fusion of transcriptional factors/DNA binding proteins and RNA binding proteins (Delattre *et al.*, 1992; Zucman *et al.*, 1993a; Sorensen *et al.*, 1994; Giovannini *et al.*, 1994; Crozat *et al.*, 1993; Rabbitts *et al.*, 1993; Prasad *et al.*, 1994b; Jeon *et al.*, 1995). These fusion proteins may deregulate the gene expression by functioning as transcriptional activators (Ohno *et al.*, 1993, 1994; May *et al.*, 1993; Bailly *et al.*, 1994).

Ewing family of tumors, which includes Ewing's sarcoma of bone, primitive neuroectodermal tumors, Askin tumors and extraskeletal Ewing's sarcoma showed recurrent t(11;22) or t(21;22) chromosomal translocations (Aurias *et al.*, 1983; Turc-Carel CA *et al.*, 1988; Delattre *et al.*, 1992; Zucman *et al.*, 1993a; Sorensen *et al.*, 1994; Giovannini *et al.*, 1994; Dunn *et al.*, 1994). Molecular analysis of these Ewing's family of tumors revealed that EWS gene on chromosome 22q12 is fused to one of two erg family members namely erg and Fli-1 genes (Reddy *et al.*, 1987; Prasad *et al.*, 1992, 1994a; Ben-David *et al.*, 1991; Watson *et al.*, 1992; Klemsz *et al.*, 1993) which are mapped to 11q24 and 21q22 (Rao *et al.*, 1987; Delattre *et al.*, 1992; Zucman *et al.*, 1993a; Sorensen *et al.*, 1994; Giovannini *et al.*, 1994; Dunn *et al.*, 1994). EWS protein is shown to function as an RNA binding protein which specifically binds to poly G and poly U (Ohno *et al.*, 1994). The conserved RGG box present in the extreme carboxy terminal region of EWS protein is shown to function as an RNA binding domain (Ohno *et al.*, 1994). The EWS-fusion domain is shown to regulate the RNA binding activity of EWS protein (Ohno *et al.*, 1994). Fli-1 and erg proteins are shown to act as sequence specific transcriptional activators with two autonomous transcriptional activation domains, one at the amino terminal region and the other at the carboxyl terminal region (Reddy and Rao, 1991; Siddique *et al.*, 1993; Rao *et al.*, 1993). We and others have shown that aberrant EWS-Fli-1 and EWS-erg proteins (found in Ewing's family of tumors) function as sequence specific transcriptional activators (Ohno *et al.*, 1993, 1994; May *et al.*, 1993; Bailly *et al.*, 1994). EWS-fusion domain functions as a regulatory/modulatory domain or as a transcriptional activation domain (Ohno *et al.*, 1993, 1994; May *et al.*, 1993; Bailly *et al.*, 1994). These aberrant proteins also

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showed altered DNA binding and transcriptional activation properties suggesting that these altered properties may be responsible for genesis of these human solid tumors (Ohno *et al.*, 1994; Mao *et al.*, 1994). Recently we have targeted the EWS-fusion products (EWS-Fli-1 and EWS-erg) of Ewing's sarcoma cells by antisense RNA technique and this resulted in a loss of tumorigenic properties of Ewing's sarcoma cells (Ouchida *et al.*, 1995). Our results also demonstrate the necessity of a certain threshold level of expression of EWS-fusion products in clonogenicity and tumorigenicity of Ewing sarcoma cells and therefore targeting EWS-fusion products may be a feasible general approach as a therapy in the cases of Ewing family tumors (Ouchida *et al.*, 1995).

TLS/FUS gene, a member of EWS family, was also shown to be fused with CHOP, in another human solid tumor myxoid liposarcoma with a t(12;16)(q13;p11) chromosome translocation (Crozat *et al.*, 1993; Rabbitts *et al.*, 1993). Molecular characterization of several types of human myeloid leukemia with a recurrent chromosome translocation t(16;21)(p11;q22) revealed fusion of TLS/FUS gene (Shimizu *et al.*, 1993; Ichikawa *et al.*, 1994; Panagopoulos *et al.*, 1994; Helm *et al.*, 1994) with erg gene, a member ets family (Reddy *et al.*, 1987; Rao *et al.*, 1987). These cases are interesting because the TLS/FUS and erg genes involved in different solid tumors (Liposarcomas and Ewing family of tumors) are fused in a hematological malignancy (human myeloid leukemia) (Panagopoulos *et al.*, 1994). Therefore it is possible that deregulation of expression of certain genes which may lead to neoplasia, may be common in both hematological and nonhematological malignancies. TLS/FUS protein functions as an RNA binding protein (Crozat *et al.*, 1993; Prasad *et al.*, 1994b) and binds specifically to poly G sequences (Prasad *et al.*, 1994b). We and others have shown that TLS/FUS-erg functions as a transcriptional activator (Prasad *et al.*, 1994b) and the TLS/FUS-fusion domain contributes transcriptional activation domain to the TLS/FUS-erg chimeric protein (Prasad *et al.*, 1994b; Sanchez-Garcia and Rabbitts, 1994). TLS/FUS-erg showed altered DNA binding and transcriptional activation functions which may be responsible for the activation of this chimeric protein in these solid tumors. Alternatively these proteins may function as sequesters by sequestering the factors that regulate the expression of genes that are critical for cell growth and differentiation.

EWS gene was also fused with another transcriptional factor Wilms tumor gene WT1 in another solid tumor, Desmoplastic small round cell tumors (DSRCT) with t(11;22)(p13;q12) chromosome translocation (Landanyi and Gerald, 1994; Gerald *et al.*, 1995).

It appears that in all these human solid tumors, RNA binding proteins (EWS or TLS/FUS) are fused with a variety of DNA binding proteins. Karyotype analysis of another rare and aggressive tumor, malignant melanoma of soft parts or soft tissue clear cell sarcoma revealed recurrent t(12;22)(q13;q12) chromosome translocation (Zucman *et al.*, 1993b). Molecular analysis of malignant melanoma of soft parts tumors and its cell lines revealed fusion between EWS gene, on chromosome 22, with ATF-1 gene, located on chromosome 12 (Zucman *et al.*, 1993b). The deduced sequence of the chimeric protein revealed fusion between amino-terminal region of EWS with nearly full length ATF-1 protein (except for 64 amino acids at the amino terminal region) (Figure 1) (Zucman *et al.*, 1993b). ATF-1 (Activating Transcription Factor) is a member of a large family of transcriptional factors ATF/CREB proteins (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). These transcriptional factors are known to activate the expression of certain genes through cis-regulatory promoter elements termed CRE (cAMP-responsive element) as a result of signals that increase intracellular cAMP (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). ATF/CREB (CRE-binding) proteins bind to these CRE (cAMP-responsive element) in a sequence specific manner (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). CREB, another member of ATF/CREB family, was characterized more thoroughly with respect to phosphorylation and its relationship with trans activation function, domains responsible for transcriptional activation, homo and heterodimerization etc (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). ATF-1 and CREB show nearly identical homology (95% identity) within the DNA

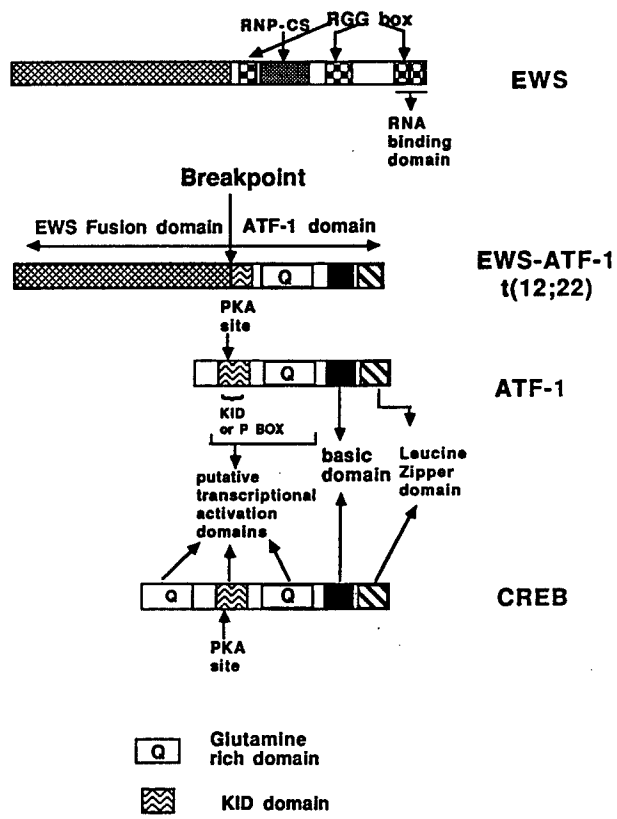


Figure 1 Schematic representation of the functional domains of the aberrant EWS-ATF-1 chimeric proteins of human malignant melanoma of soft parts and comparison with normal ATF-1 and EWS proteins. Carboxy-terminal region containing RGG box functions as a RNA binding domain of EWS protein (Ohno *et al.*, 1994). Putative transcriptional activation domains (KID + Q domain) of ATF-1/EWS-ATF-1 are also shown (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991; Brindle *et al.*, 1993). RNP-CS represents the domain conserved in a variety of RNA binding proteins. Basic and Leucine zipper domains responsible for DNA binding and dimerization are shown. Protein kinase A (PKA) site of ATF-1 is shown. This PKA site is lost in EWS-ATF-1 as a result of translocation

binding domain which includes leucine-zipper domain involved in homo and hetero-dimerizations and the basic domain responsible for interacting with DNA (Figure 1). In addition, ATF-1 and CREB proteins show significant overall homology (70%) throughout the coding region except in the amino terminal region (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). Both these proteins conserved the phosphorylation site (PKA site) for protein kinase A and Ca2+/calmodulin-dependent kinase I and II and two other regions termed PDE-1 and PDE-2 flanking this PKA site (KID or P box) (Figure 1). The transcriptional activation domain of CREB consists of several independent domains which include KID (Kinase Inducible Domain) domain and the two glutamine rich (Q) domains flanking this KID domain (Brindle *et al.*, 1993) (Figure 1). Unlike CREB, ATF-1 contains only the carboxy-terminal glutamine rich domain (Figure 1). KID domain contain several consensus phosphorylation sites for various kinases including PKA, PKC and Ca2+/calmodulin-dependent kinase I and II (Figure 1). The transactivation glutamine-rich domain (Q domain) present in the carboxy terminal region of CREB proteins shows high homology with ATF-1 suggesting that this glutamine rich domain present in the carboxy terminal region of ATF-1 may also function as a transcriptional activation domain (Figure 1).

In order to understand the mechanism of activation of aberrant EWS-ATF-1 in malignant melanoma of soft parts, we have cloned the cDNA representing the normal and aberrant full length EWS-ATF-1 transcripts. In this report, we have studied the DNA binding and transcriptional activation properties of EWS-ATF-1 chimeric protein. Our results demonstrate that EWS-ATF-1 functions as a constitutive transcriptional activator unlike the normal ATF-1. We also show that the EWS-fusion domain functions as a

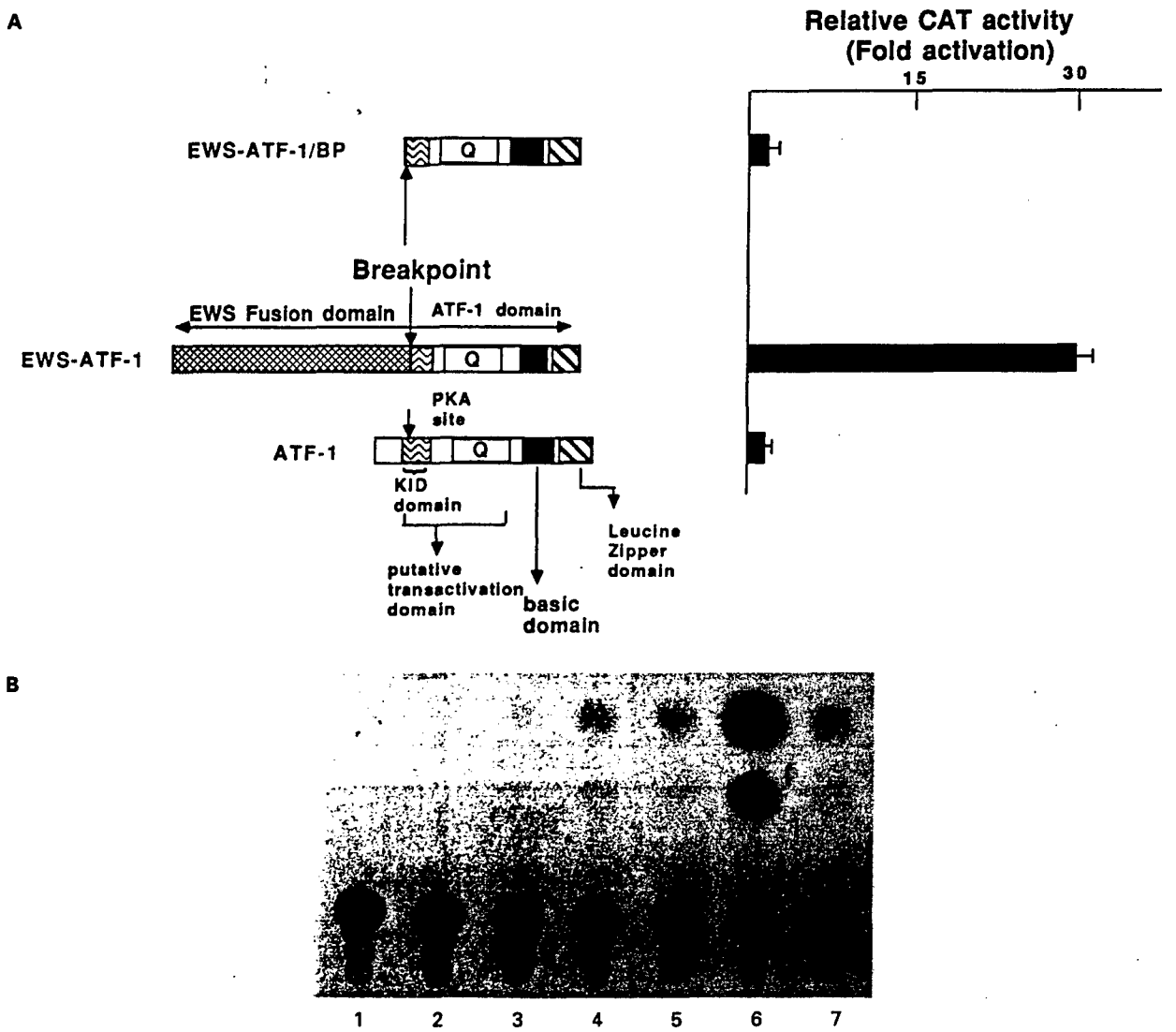


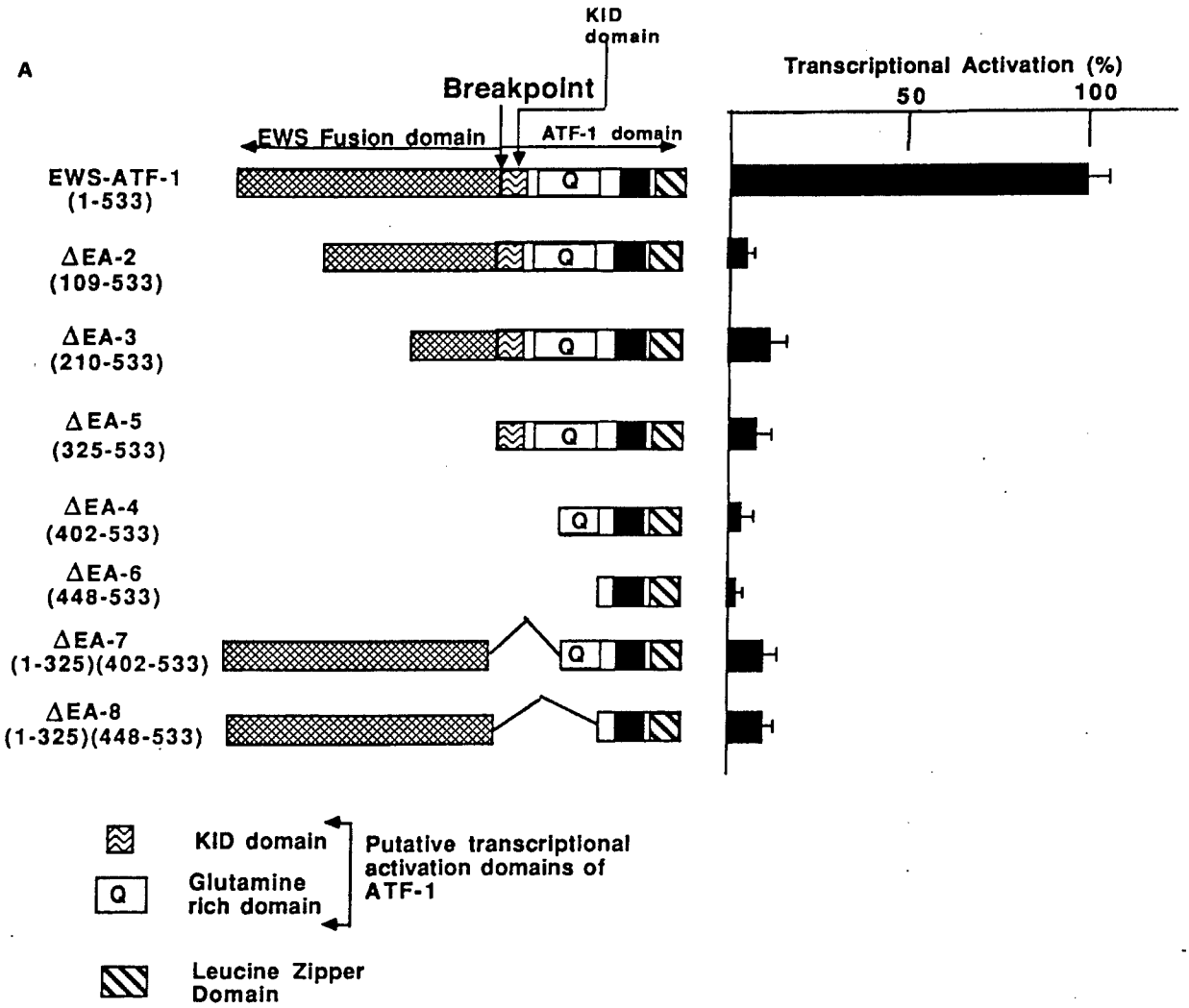
Figure 2 EWS-ATF-1 chimeric protein is a constitutive transcriptional activator. (**A**) Schematic representation of the constructs containing normal and aberrant ATF-1 cDNAs in pSG5 vector. EWS-ATF-1/BP represents the construct where amino terminal region of EWS is deleted from EWS-ATF-1. Transcriptional activation of these normal, aberrant and amino-terminal truncated ATF-1 proteins were studied by cotransfection of these expression plasmids with reporter (SOM-CAT) and reference plasmids in QT6 cells. Fold activation of transcription for various constructs (compared to vector control) is shown. Each bar represents the normalized values for the mean ±SD of five independent experiments. (**B**) The chromatogram represents a typical transfection. Lane 1, pBL CAT3+pSG5; Lane 2, pBL CAT3+ATF-1; Lane 3, pBL CAT3+EWS-ATF-1; Lane 4, SOM-CAT+pSG5; Lane 5, SOM-CAT+ATF-1; Lane 6, SOM-CAT+EWS-ATF-1; Lane 7, SOM-CAT+EWS-ATF-1/BP

regulatory domain and regulates the transcriptional activation properties of EWS-ATF-1 fusion protein. We demonstrate that protein-protein interactions through leucine zipper domain of chimeric protein plays an important role in transcriptional activation. Sequestering of the transcriptional factors (CBP, p300 etc) by EWS-ATF-1 proteins could be one of the mechanisms of activation of EWS-fusion proteins in human sold tumors.

Results and discussion

EWS-fusion domain is needed for constitutive transcriptional activation by EWS-ATF-1 chimeric protein

ATF-1 was shown to bind to DNA (CRE, cAMP-response element) in a sequence specific manner (Lee and Masson, 1993; Liu *et al.*, 1993) and also to function as a transcriptional activator only after induction with cAMP inducers (Lee and Masson, 1993; Liu *et al.*, 1993). To test whether EWS-ATF-1 functions as a transcriptional activator, we have made a reporter plasmid by cloning somatostatin promoter (carrying cAMP -response element) upstream of the CAT gene and used this reporter plasmid (SOM-CAT) to study transcriptional activation properties of ATF-1 and EWS-ATF-1. Expression of ATF-1, EWS-ATF-1 and truncated ATF-1 (EWS-ATF-1/BP, a plasmid expressing the ATF-1 domain of EWS-ATF-1 chimeric protein) was obtained by cloning full length ATF-1, EWS-ATF-1 and truncated ATF-1 into pSG5 vector (Figure 2). As expected ATF-1 showed no transcriptional activation in the absence of cAMP induction (Figure 2). However EWS-ATF-1 showed potent transcriptional activation (30-fold more compared to vector control) (Figure 2). EWS-ATF-1/BP (without EWS-fusion domain) failed to show transactivation with SOM-CAT reporter (Figure 2). We expect this result because truncated ATF-1 lacks the phosphorylation site which is essential for transactivation function (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). These results suggest that EWS-ATF-1 functions as a constitutive transcriptional activator even in the absence of cAMP induction unlike normal ATF-1. To rule out the possibility that EWS-ATF-1 is a general transcriptional activator, we have tested the effect of EWS-ATF-1 chimeric protein on transactivation of reporter plasmids lacking the ATF-1 binding site (Figure 2B). The reporter plasmid was not transactivated by EWS-ATF-1 (Figure 2) demonstrating that ATF-1 binding site is required for efficient transactivation of reporter plasmids. We have



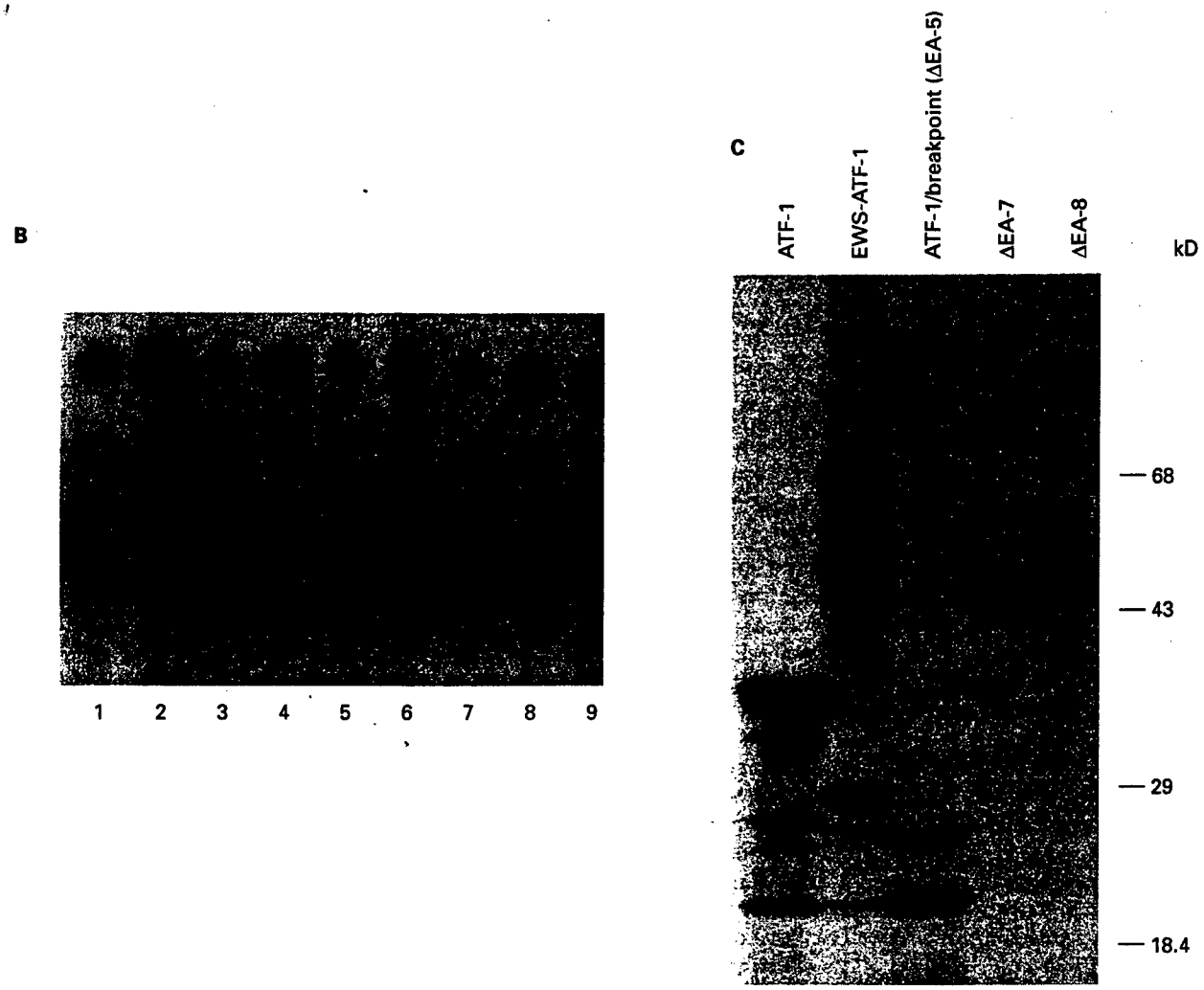


Figure 3 (A) EWS-fusion domain functions as a regulatory domain for the transactivation properties of EWS-ATF-1 chimeric protein. Schematic representation of the various mutants of EWS-ATF-1 (in pSG5 vector) used for studying the transactivation properties.QT6 cells were cotransfected with each of these various EWS-ATF-1 mutants, reporter plasmid (SOM-CAT) and a reference plasmid as in Figure 2. Transcriptional activation of EWS-ATF-1 is taken as 100%. Each bar represents the normalized values for the mean ± SD of five independent experiments. (B) The chromatogram represents a typical transfection. Lane 1, SOM-CAT+pSG5; lane 2, SOM-CAT+EWS-ATF-1; lane 3, SOM-CAT+EA-2; lane 4, SOM-CAT+EA-3; lane 5, SOM-CAT+EA-5; lane 6, SOM-CAT+EA-4; lane 7, SOM-CAT+EA-6; lane 8, SOM-CAT+EA-7; lane 9, SOM-CAT+EA-8. (C) *In vitro* transcription and translation of ATF-1, EWS-ATF-1 and its deletion mutants. *In vitro* transcription and translation was carried as described in Materials and methods

also tested the effect of EWS-ATF-1 mutant (in-frame deletion lacking the basic DNA binding domain) on the transactivation of SOM-CAT reporter plasmid. Our results demonstrate that this mutant of EWS-ATF-1 failed to transactivate the reporter plasmid (data given in Figure 5). These results suggest that EWS-ATF-1 codes for sequence specific constitutive transcriptional activator. We have also studied the effect of forskolin (an activator of adenyl cyclase) on the transcriptional activation properties of EWS-ATF-1 using SOM-CAT reporter plasmid. Our results demonstrate no dramatic effect on the transcriptional activation function of EWS-ATF-1 (data not shown).

EWS-fusion domain functions as a regulator/modulator domain for the transcriptional activation properties of EWS-ATF-1 chimeric protein

EWS-fusion domain of EWS-Fli-1/EWS-erg chimeric proteins was shown to function either as a regulatory/modulatory or transcriptional activation domain (Ohno *et al.*, 1993, 1994; May *et al.*, 1993; Bailly *et al.*, 1994). In order to know whether EWS-fusion domain of EWS-ATF-1 functions as a modulatory or transcriptional activation domain, we have made a series of deletion mutants and tested their transactivation function using SOM-CAT reporter plasmid. Deletion of aminoterminal domain up to amino acid 109 (ΔEA-2) showed significant loss of transcriptional activation of SOM-CAT reporter (Figure 3A and 3B). As expected constructs expressing amino-terminal truncated EWS-ATF-1 proteins with deletions extending to amino acids 210 (ΔEA-3) and aminoacid 325 (ΔEA-5) of EWS-fusion domain showed no significant transcriptional activation function suggesting that amino-terminal region of EWS-fusion domain is required for transcriptional activation function by EWS-ATF-1 chimeric protein (Figure 3A and 3B). Amino-terminal deletion mutants ΔEA-4 and ΔEA-6 also showed no transcriptional activation domain. Since ATF-1 domain of EWS-ATF-1 chimeric protein contain transcriptional activation motifs (KID + Q,

glutamine rich domain) (Figure 1), it is not certain whether EWS-fusion domain contributes to the transcriptional activation domain or modulates the transcriptional activation function of ATF-1 transcriptional activation domains (KID and Q domains) (Figure 3A and 3B). To test these possibilities, we have made internal deletion of EWS-ATF-1 chimeric protein in such a way that putative transactivation domains of ATF-1 were deleted either partly or completely in EWS-ATF-1 constructs (ΔEA-7 and ΔEA-8). The reading frame of these constructs were verified by *in vitro* transcription and translation (Figure 3C) and tested for transactivation function on SOM-CAT reporter plasmid. Our results show that both deletion mutants (ΔEA-7 and ΔEA-8) failed to show transactivation function (Figure 3A and 3B). These results suggest that EWS-fusion domain of EWS-ATF-1 failed to show transactivation function in the absence of putative ATF-1 transactivation domains (KID + Q domain) present in the fusion protein. From these results one can conclude that EWS-fusion domain functions as a modulatory/regulatory domain for the transactivation properties of EWS-ATF-1 chimeric protein. It appears that EWS-fusion domain regulates transcriptional activation properties of the KID domain of EWS-ATF-1 (comparing the CAT activities of EWS-ATF-1 and ΔEA-7, Figure 3A and B). Similar regulatory functions were observed earlier with EWS-fusion domain in the case of EWS-erg and EWS-Fli-1 proteins (Ohno *et al.*, 1993, 1994). EWS-fusion domain of EWS-Fli-1/EWS-erg was also shown to function as a transcriptional activation domain in a heterologous system and also using different reporter plasmids (May *et al.*, 1993; Bailly *et al.*, 1994). Analysis of transactivation properties of various deletion mutants of EWS-ATF-1 revealed that amino-terminal region up to aa 109 of EWS-fusion domain plays a major role in the regulatory function of EWS-ATF-1 protein and therefore, this region (aa1–109) of EWS-fusion domain represents regulatory domain of EWS-ATF-1 protein. These results are different from what we have observed with EWS-Fli-1 and EWS-erg proteins, where amino-terminal deletion up to aa 210 of EWS-fusion domain has no major effect on transactivation properties of EWS-Fli-1 and EWS-erg chimeric proteins (Ohno *et al.*, 1993, 1994). Therefore it appears that different regions of EWS-fusion domain are needed for the regulation of transcriptional activation depending on the fusion partner (Fli-1, erg, ATF-1 etc), reporter plasmids and protein-protein interaction (displayed in different cell types).

EWS-fusion domain functions as a negative regulatory domain for the DNA binding properties of EWS-AFT-1 chimeric protein

We have also compared the sequence specific DNA binding properties of aberrant EWS-ATF-1 and normal ATF-1 by electrophoretic mobility shift assay (EMSA) (Ohno *et al.*, 1993, 1994) using SOM promoter fragment (data not shown) or oligonucleotide containing ATF-1/CREB target sequence (Figure 4). EMSA was carried out with equal amount (as judged by band intensity on SDS–polyacrylamide gel and also taking methionine content into consideration) of *in vitro* translated EWS-ATF-1, ATF-1, CREB and

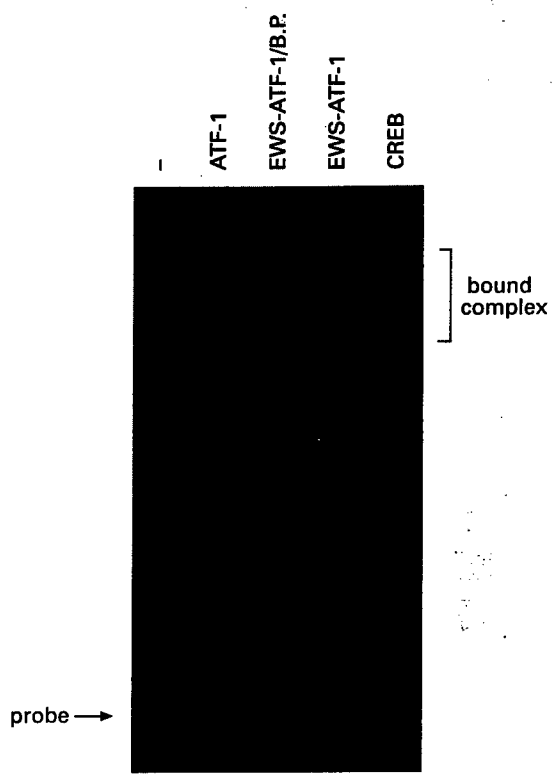


Figure 4 Sequence specific DNA binding properties of EWS-ATF-1, ATF-1 and EWS-ATF-1/BP (with deletion of EWS-fusion domain) (please see Figure 2) and CREB proteins. Electrophoretic mobility shift assay (EMSA) was carried out with ^{32}P-labelled oligonucleotide containing ATF-1 target sequence and *in vitro* translated proteins (equal quantity of protein as judged by the intensity of the band and methionine content was used). The bound nucleo-protein complexes were resolved on 6% PAGE and visualized by autoradiography. Lane 1, minus RNA; Lane 2, ATF-1; Lane 3, EWS-ATF-1/BP (with a deletion of EWS-fusion domain); Lane 4, EWS-ATF-1 and Lane 5, CREB

^{32}P-labelled SOM promoter or ATF1/CREB target sequence oligonucleotide as a probe. Under the same conditions where normal ATF-1 showed strong nucleoprotein complex, EWS-ATF-1 showed weak nucleoprotein complex (Figure 4). These results are surprising taking into consideration that EWS-ATF-1 contain intact ATF-1 DNA binding domain and also EWS-ATF-1 functions as an efficient transcriptional activator. We have also observed such a loss of DNA binding activity with TLS/FUS-erg fusion protein even though erg DNA binding domain is intact in TLS/FUS-erg (Prasad *et al.*, 1994). It is possible that EWS-fusion domain may be acting as a negative regulatory domain for the DNA binding activity of EWS-ATF-1. EWS-ATF-1 protein may bind to target sequences *in vivo* in cooperation with some other factors and thereby function as an efficient transcriptional activator. Such an observation was seen in the case of elk-1 (Rao *et al.*, 1989) where we observed that elk-1 binds to DNA (serum response element, SRE) only after interaction with serum response factor (SRF) (Hipskind *et al.*, 1991; Rao and Reddy, 1992). Leucine-zippers are shown to be responsible for homo- and hetero-dimerization of ATF-1 and is also needed for transactivation function of ATF-1 (Lee and Masson, 1993; Liu *et al.*, 1993). In order to test whether such

protein-protein interactions (dimerization) are needed for transactivation of EWS-ATF-1 chimeric protein, we tested the transactivation of EWS-ATF-1 construct that lacked the leucine-zipper domain (ΔLZ-EWS-ATF-1) (Figure 5A and 5B). ΔLZ-EWS-ATF-1 failed to transactivate the SOM-CAT demonstrating that leucine zipper motif is essential for transactivation function of EWS-ATF-1 (Figure 5A and B). These results also suggest that protein-protein interactions through the leucine-zipper motif may play an important role in the transactivation function. As expected EWS-ATF-1 construct lacking the basic domain (ΔB-EWS-ATF-1) failed to transactivate the SOM-CAT reporter demonstrating requirement of DNA binding activity for transcriptional activation (Figure 5A and 5B).

CREB and ATF-1 proteins are activated as a result of phosphorylation by protein kinase A (PKA) (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). This phosphorylation appears to have no major effect on the DNA binding activity to CRE (cAMP Response Element) and also on the intracellular level of CREB/ATF-1 proteins (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). However

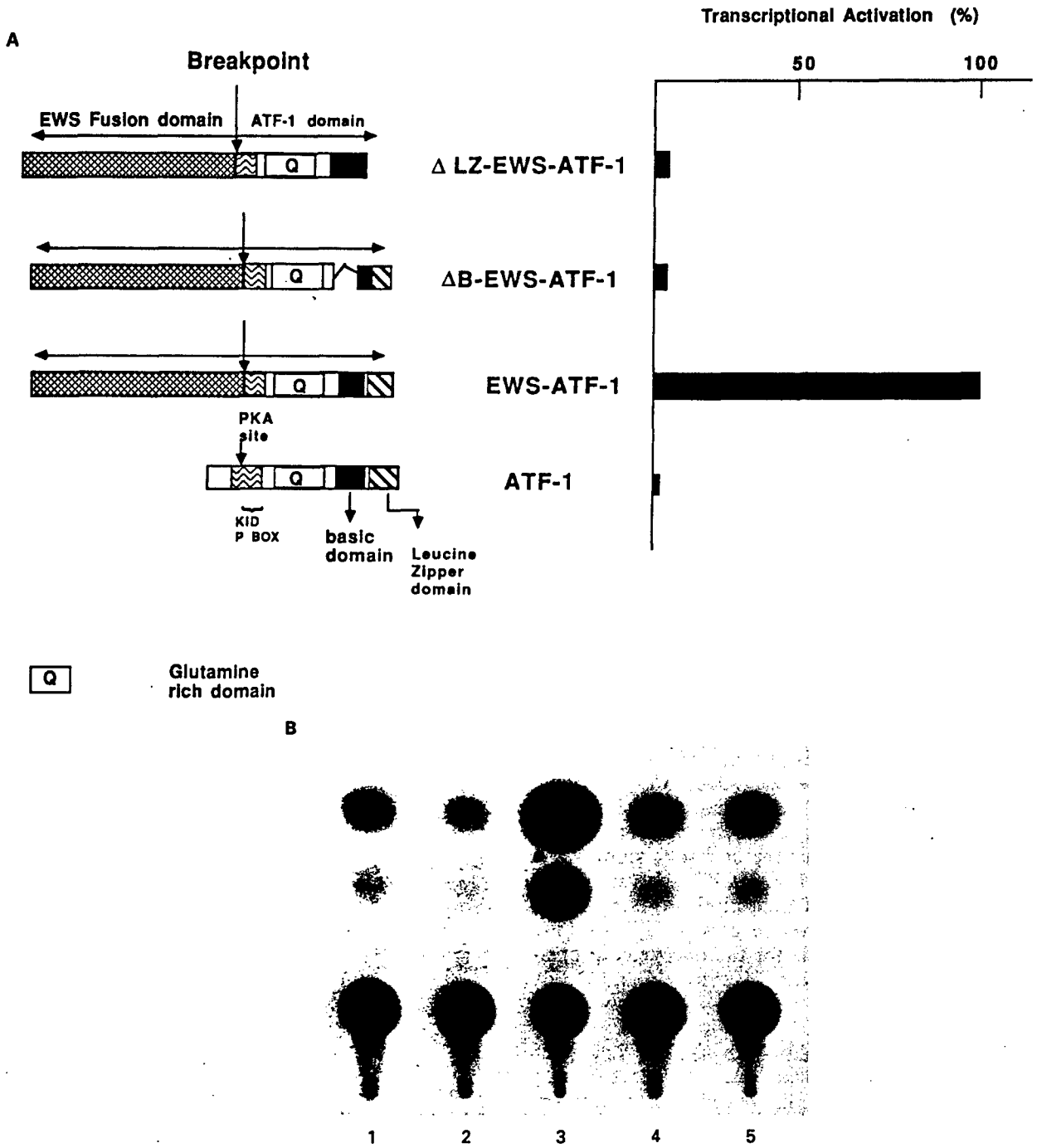


Figure 5 (**A**) Protein-protein interaction through leucine-zipper domain plays an important role in the transcriptional activation properties of EWS-ATF-1 fusion protein. Schematic representation of the constructs used for studying the role of leucine-zipper and basic domains in transcriptional activation properties of EWS-ATF-1 are shown. QT6 cells were transfected with reporter plasmid (SOM-CAT), pCH110 (reference plasmid) along with expression plasmids containing various deletion mutants of EWS-ATF-1 described in Figure 2. (**B**) The chromatogram represents a typical transfection. Lane 1, SOM-CAT+pSG5; lane 2, SOM-CAT+ATF-1; lane 3, SOM-CAT+EWS-ATF-1; lane 4, SOM-CAT+ΔLZ-EWS-ATF-1; lane 5, SOM-CAT+ΔB-EWS-ATF-1

transcription of CRE driven genes were enhanced greatly as a result of phosphorylation (Lee and Masson, 1993; Liu *et al.*, 1993; Gonazalez *et al.*, 1991). Recent results suggested that only phosphorylated (but not underphosphorylated) CREB recruits/binds to transcriptional co-activator CBP (CREB Binding Protein) (Chrivia *et al.*, 1993). This transcriptional co-activator CBP was shown to interact with basal transcriptional factor TFIIB through its carboxy-terminal transactivation domain (Kwok *et al.*, 1994). This TFIIB in turn interacts with the TBP (TATA box binding protein) and participates in the recruitment of RNA polymerase holoenzyme to the promoters resulting in the transcriptional activation (Kwok *et al.*, 1994). In the case of mitogen inducible transcription, c-Jun is also shown to recruit CBP (Arias *et al.*, 1994). Therefore it appears that signals from cell surface receptors are relayed to transcriptional system through phosphorylated transcriptional factors (such as ATF-1/CREB family members etc) and their transcriptional coactivators resulting in the regulation of cell growth and differentiation. It is possible that aberrant transcriptional factors (such as ESW-ATF-1) may short circuit this cell surface signal transmission by its altered DNA binding, protein-protein interactions and/or transcriptional activation functions resulting in the deregulated cell growth and differentiation leading to neoplasia (Vairo *et al.*, 1990; Kato *et al.*, 1994; Jones *et al.*, 1994; Boshart *et al.*, 1991; Arany *et al.*, 1995; Lundblad, 1995). EWS-ATF-1 chimeric proteins may bind directly to transcriptional co-activators/factors (without being regulated by phosphorylation) and activate transcription of critical genes involved in cell growth and/or apoptosis. Alternatively these aberrant EWS-ATF-1 proteins may sequester critical transcriptional factors that participate in gene regulation resulting in the interference in cAMP pathway which is known to be responsible for growth-inhibitory and differentiation (Vairo *et al.*, 1990; Kato *et al.*, 1994; Jones *et al.*, 1994; Boshart *et al.*, 1991; Arany *et al.*, 1995; Lundblad, 1995). In this sequestering phenomenon, other CREB/ATF family members may be deprived of transcriptional co-factors/co-activators resulting in the inhibition of various promoters containing CREs, whose activity are critical for differentiation (Jones *et al.*, 1994; Boshart *et al.*, 1991). Therefore EWS-ATF-1 may be inhibiting differentiation by targeting the transcriptional factors such as CBP, p300 etc. Our recent results show direct interaction of EWS-ATF-1 and CBP suggesting that constitutive sequestering of transcriptional factors such as CBP/p300 by the over expressed EWS-ATF-1 proteins may be responsible for the oncogenic properties of EWS-ATF-1 (Fujimura Y, Rao VN and Reddy ESP unpublished results). Recently E1A onco-protein was shown to target a family of transcriptional adaptor/co-activator proteins such as CBP and p300 (Arany *et al.*, 1995; Lundblad *et al.*, 1995) which may be responsible for E1A induced cell immortalization. Similarly, viral proteins (SV 40 T antigen, E1B etc) are known to bind p53 and interfere with its function. Thus viral transforming proteins and fusion onco-proteins appear to follow similar strategy (function as sequesters) for transformation of cells. It is also possible that both transcriptional activation and sequestering properties of EWS-ATF-1 may play a role in transformation. Therefore further studies on the role of DNA binding, transactivation and protein-protein interactions in the oncogenic potential of the aberrant EWS-ATF-1 proteins are critical for understanding neoplasia of these solid tumors and for future development of therapeutic agents. Recently, Brown *et al.* (1995) have also shown that EWS-ATF-1 functions as a constitutive transcriptional activator.

Materials and methods

Molecular cloning of normal and aberrant ATF-1 cDNAs and the construction of their mutants

cDNA was obtained by reverse transcription of total RNA from various cells (Colo 320, Molt4, KG1, SU-CCS-1 etc) using cDNA kit (Boehringer Mannheim). ATF-1 cDNA was amplified using appropriate 5′ and 3′ primers as described (Ohno *et al.*, 1993). Similarly EWS-ATF-1 and its deletion mutants were obtained by polymerase chain reaction using appropriate 5′ and 3′ primers and by utilizing convenient restriction sites. EWS-ATF-1 and ATF-1 cDNAs and their truncated fragments were subcloned into pSG5 vector. Appropriate initiation and termination codons were introduced where needed. These constructs were characterized by restriction mapping and nucleotide sequencing. SOM-CAT reporter plasmid was constructed by cloning the PCR amplified somatostatin promoter (Bgl 11 to Xba 1) into pBL CAT3 vector.

Transcriptional activation studies of EWS-ATF-1 and ATF-1 and their mutants

QT6 cells were cotransfected with 1μg of expression plasmid, 1 μg of reporter plasmid and 5 μg of reference plasmid (pCH110) using calcium phosphate method as described previously (Ohno *et al.*, 1993). CAT and β-galactosidase activities were carried out as described (Ohno *et al.*, 1993).

In vitro *transcription and translation*

Expression plasmids containing appropriate cDNAs were linearized and *in vitro* transcribed and translated as described (Ohno *et al.*, 1994).

Electrophoretic mobility shift assays (EMSA)

EMSA was carried out with *in vitro* translated EWS-ATF-1, ATF-1 and their mutant proteins using ^{32}P-labelled ATF-1 target sequences (oligonucleotide E79, GATCCATGACGTCATG) or somatostatin promoter fragment as described (Liu *et al.*, 1993). The bound nucleoprotein complexes were competed with 50 fold molar excess of cold competitor (E79) but not with nonspecific competitor suggesting that ATF-1, EWS-ATF-1 and CREB bind to DNA in a sequence specific manner (data not shown).

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Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts

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Recently, BRCA1, a familial breast and ovarian cancer susceptible gene has been cloned and shown to be either lost or mutated in families with breast and ovarian cancers. BRCA1 has been postulated to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth. We have characterized the BRCA1 gene products by Western blot and immunoprecipitation analysis in mouse and tumor cells. Multiple BRCA1 polypeptides of approximately 225, 185, 160, 145, 100, 52 and 38 kD were identified in these cells. BRCA1 proteins were found to be localized mainly in the nucleus of normal Rat1 cells and human breast cancer cells. In order to understand the role of BRCA1 in cell transformation, we have established a stable NIH3T3 cell line expressing BRCA1 antisense RNA. The inhibition of expression of endogenous BRCA1 protein was detected in NIH3T3 transfectants by Western blot analysis. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental and sense transfectants. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene.

Keywords: BRCA1 proteins; antisense RNA; tumor suppressor; breast and ovarian cancers

Introduction

Breast cancer is one of the most frequent cancers affecting women. Although majority of cases are thought to be sporadic, about 5% of cases are estimated to be familial (Claus *et al.*, 1991). The clinical progression of human breast cancer reflects accumulated molecular defects in specific genes that are important in regulating the growth of normal breast tissue. The breast cancer susceptible gene BRCA1 gene was shown to be lost or mutated in families with hereditary breast and ovarian cancers (Miki *et al.*, 1994; Takanashi *et al.*, 1995; Easton *et al.*, 1993). Some recent reports have also implicated a role for BRCA1 directly in sporadic cancers (Merajver *et al.*, 1995; Hosking *et al.*, 1995; Futreal *et al.*, 1994). Studies of allele loss in tumors from breast and ovarian cancer affected families suggesting that BRCA1 is a tumor suppressor gene (Smith *et al.*, 1992). Thus the inherited mutation results in inactivation of one copy of the gene by mutation and the loss of the second wild type allele (Smith *et al.*, 1993; Kelsell *et al.*, 1993). These results implicate a key role for tumor suppressor genes like BRCA1 in the genesis and progression of breast cancers. The BRCA1 gene is composed of 22 coding exons stretching roughly 100 Kb of genomic DNA (Miki *et al.*, 1994). The gene codes for a 1863 amino-acid protein with an amino terminal zinc finger domain and a carboxy terminal acidic region typical of several transcriptional factors (Miki *et al.*, 1994). The first insight into the potential role of BRCA1 in breast tumor progression came from the work described by Thompson *et al.* (1995) where they show inhibition of BRCA1 expression with antisense oligonucleotides resulted in accelerated growth of normal and malignant mammary cells but not non-mammary epithelial cells. These results suggested that BRCA1 negatively regulates the proliferation of mammary epithelial cells. Taking all these results into consideration, we reasoned that if BRCA1 functions as a tumor suppressor regulating cell growth and division, allelic loss or damage by mutation of BRCA1 as seen in patients with breast cancer could result in loss of function of BRCA1 protein and uncontrolled cell growth leading to cancers. To test the hypothesis whether BRCA1 is a tumor suppressor gene, we used antisense RNA methodolgy (Iszat and Weintraub, 1985). We reasoned that if BRCA1 functions as a growth regulator in normal cells, inhibiting its expression should result in transformation. Our results demonstrate that antisense RNA to BRCA1 transforms mouse fibroblasts, providing the first direct biological evidence for the possible function of BRCA1, as a tumor suppressor gene.

Results and discussion

The experimental strategy that we have used to test the hypothesis whether BRCA1 is a tumor suppressor gene is shown in Figure 1. NIH3T3 cells express significant levels of a major ≈100 kD and a minor ≈145 kD BRCA1 protein as analysed by Western blot analysis (Figure 2a, lane 1). Similar sized BRCA1 proteins were also observed in several human cell lines (Figure 2c). In addition to these bands, we have also observed both higher ≈225 kD (using nuclear extracts, data not shown), 185 kD (Figure 2c, lane 3), ≈160 kD (data not given) and lower (≈52 and ≈38 kD) molecular weight BRCA1 polypeptides (Figure 2c). Interestingly, we have detected high level of expression of ≈185–200 kD and ≈38kD BRCA1 proteins in HL 60 cells by immunoprecipitation analysis (Figure 2e). These results suggest that Western blot and immunoprecipitation analysis detect different size and level of expression of BRCA1 proteins. Detection of

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different size BRCA1 proteins is consistent with the presence of alternatively spliced transcripts of BRCA1 in different cells (Miki *et al.*, 1994; Rao, unpublished results). Our results suggest that these BRCA1 proteins undergo phosphorylation (Rao, unpublished results). We have isolated and characterized a BRCA1 cDNA corresponding to ≈100 kD BRCA1 protein in human cells (Rao, unpublished results). It remains to be seen whether these multiple protein bands represent other isoforms of BRCA1 or its related proteins. We have studied the subcellular distribution of BRCA1 proteins in normal (Rat 1 cells, Figure 2d) and in transformed cells (MCF7 cells, Figure 2d) using immunohistochemical methods (Figure 2d) and indirect immunofluorescense staining (data not given). Our results suggest that BRCA1 proteins are localized mainly in the nucleus of Rat 1 and MCF7 cells. However we have also observed weak cytoplasmic staining in the case of MCF7 cells.

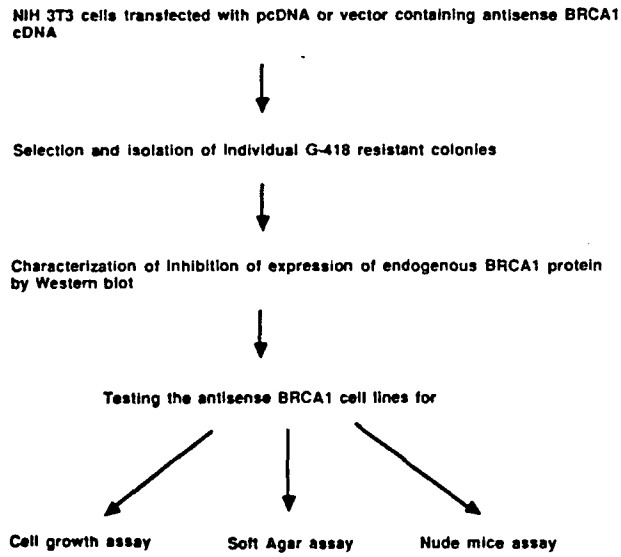
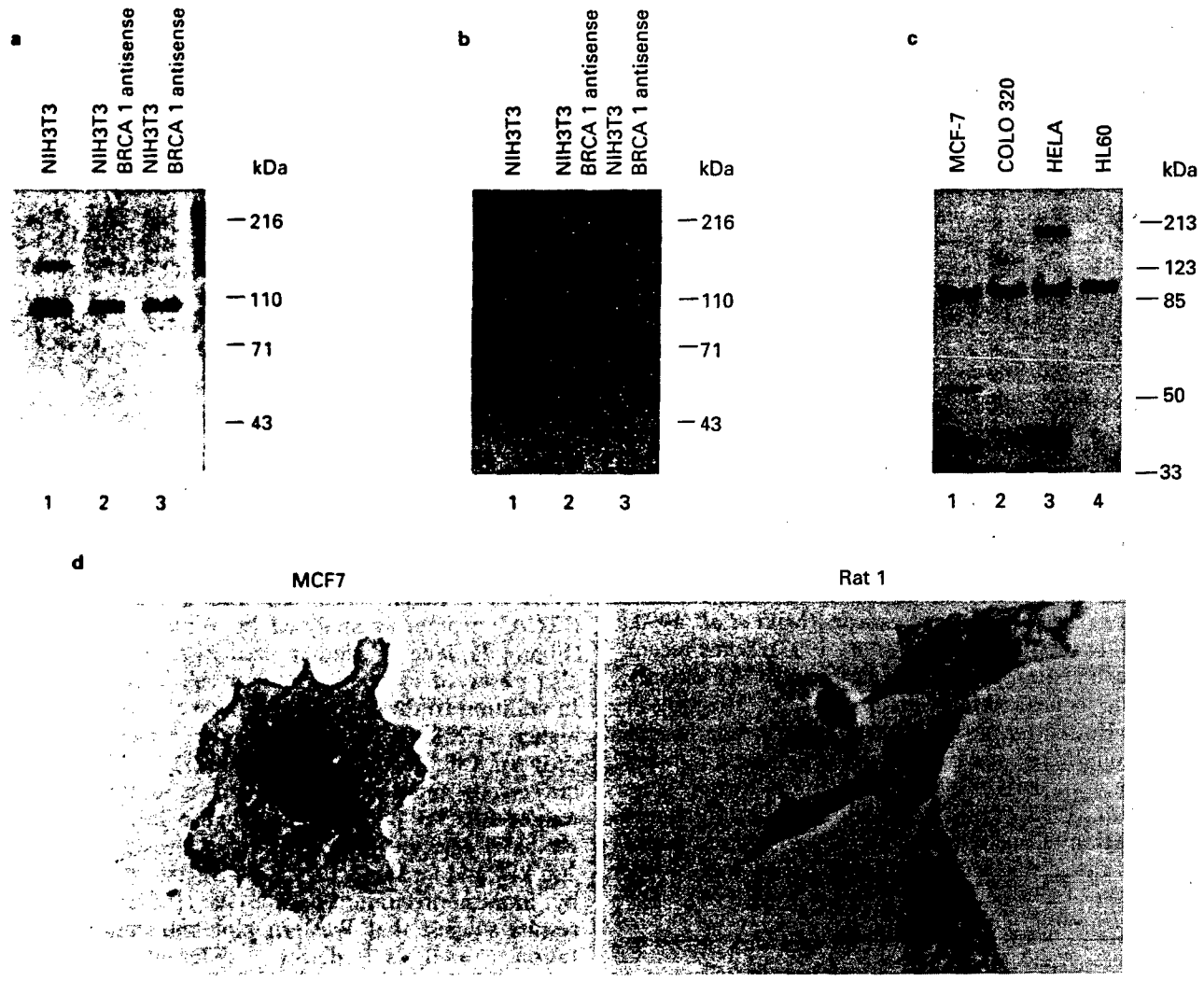


Figure 1 Experimental strategy used for obtaining the antisense BRCA1 transfectants

We have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vectors containing antisense BRCA1 cDNA and obtained stable G418 resistant cell lines expressing antisense RNA to BRCA1. Initially we have screened several BRCA^{AS} cell lines for BRCA1 protein expression by Western blot analysis using BRCA1 polyclonal peptide antibody. We selected two cell lines which showed a significant decrease in the expression of BRCA1 protein (Figure 2a, lanes 2 and 3). These lines showed roughly 3–5-fold decrease in the expression of BRCA1



protein when compared to the parental NIH3T3 cells. We have performed BRCA1 peptide competition to show that the protein band(s) seen on the immunoblot are specific for the BRCA1 protein(s) (Figure 2b). We did not find total inhibition of expression with BRCA^{AS} transfectants because in theory, a high concentration of antisense RNA is necessary to completely inhibit any target gene, however, our results (discussed below) suggests that total inhibition may not be necessary to observed a biological change, since mRNA molecule can synthesize several copies of protein.

The BRCA^{AS} transfectants showed no major morphological alterations except for a slightly more flattened phenotype when compared to the parental NIH3T3 cells (compare Figure 3a and d–f). Transformed cells unlike normal cells can proliferate faster and grow in serum-free or low serum culture medium as they become independent of growth factors present in the serum. Thus we investigated the growth of BRCA1^{AS} transfectants in different serum culture conditions (10%, 0.1% and serum free). The BRCA1^{AS} transfectants proliferated at a much faster rate than NIH3T3 cells and also the BRCA1^{AS} cells proliferated in low serum media (Figure 3e and 4a and b) and serum free media (Figure 3f), whereas the parental NIH3T3 cells were unable to proliferate under these conditions (Figures 3b, c and 4b). These data indicate that BRCA1^{AS} transfectants behave like transformed cells in that they become independent of growth factors present in fetal bovine serum.

The accelerated growth rate and growth in serum free media of the BRCA1^{AS} cells raised the possibility that they might have become transformed, hence we tested their ability to grow in soft agar. Interestingly the BRCA1^{AS} cells were anchorage independent (Figure 5a) unlike the parental NIH3T3 cells and cells transfected with the BRCA1 sense constructs (Figure 5a). BRCA1^{AS} transfectant cell line #6 showed high clonogenic affinity (average 150 colonies, Figure 5b) in soft agar assay whereas NIH3T3 cells, NIH3T3/pcDNA and NIH3T3/BRCA1^{S} transfectants cells showed no colonies (Figure 5b). Another BRCA1^{AS} cell line number 3 also showed accelerated growth rate and growth in soft agar but was less tumorigenic than BRCA1^{AS} cell line no. 6 (data not given). Our results clearly suggests that a certain threshold level of BRCA1 protein is required for the regulation of cell growth in both mammary epithelial cells (Thompson *et al.*, 1995) and mouse fibroblasts (this study). Mere down regulation of expression of BRCA1 protein may result in deregulation of BRCA1 function leading to the progression from a normal to a transformed state. These results demonstrate that inhibition of expression of BRCA1 protein in BRCA1^{AS} cells might be sufficient to achieve transformation.

We next tested the tumorigenicity *in vivo* of NIH3T3 cells transfected with either pcDNA vector to BRCA1 antisense cDNA in nude mice. Our results show that subcutaneous injection of BRCA1^{AS} cells into nude mice consistently resulted in the development of tumors at the site of injection with a latency of 3 to 4 weeks (six out of six animals). None of the mice injected with the vector transfectants induced tumors at least up to 6 weeks.

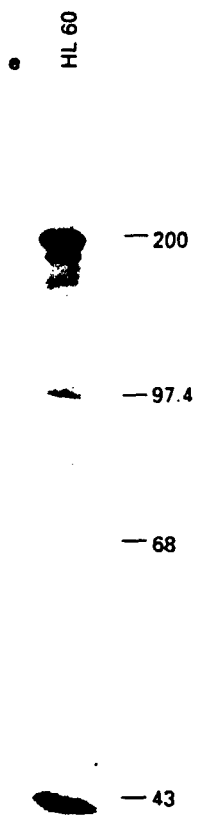


Figure 2 Characterization and subcellular localization of BRCA1 proteins. (**a**) Western blot analysis of total cellular BRCA1 protein levels in NIH3T3 and BRCA^{AS} transfectants. Total cellular extracts from NIH3T3 cells (lane 1), BRCA1 AS cell line no 3 (lane 2) and BRCA^{AS} cell line no 6 (lane 3) were electrophoretically separated, transferred to PVDF membrane and subjected to immunoblot analysis using a BRCA1 antipeptide antibody (Santacruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weight of the major BRCA1 protein band corresponds to $\approx$100 kD. (**b**) The same blot as in (**a**) was stripped and reprobed with the BRCA1 peptide antibody but in the presence of excess BRCA1 antigen. (**c**) Total cellular extract ($\approx$70 μg) from MCF-7 cells (human breast cancer cells) (lane 1); Colo 320 (human colon adenocarcinoma) (lane 2); HeLa cell (human epitheloid carcinoma) (lane 3); HL-60 (human promyelocytic leukemia) ($\approx$35 μg) (lane 4) were subjected to Western blot analysis as described in (**a**). (**d**) Immunohistochemical detection of BRCA1 proteins in normal and breast cancer cells. In Rat1 cells BRCA1 is localized only to nucleus and in MCF7 cells BRCA1 is localized mostly in nucleus. Original magnification $\times$400. (**e**) Immunoprecipitation of BRCA1 proteins in HL60 cells. HL60 cells were labelled with [^{35}S] trans label and subjected to immunoprecipitation using a carboxy terminal BRCA1 peptide antibody

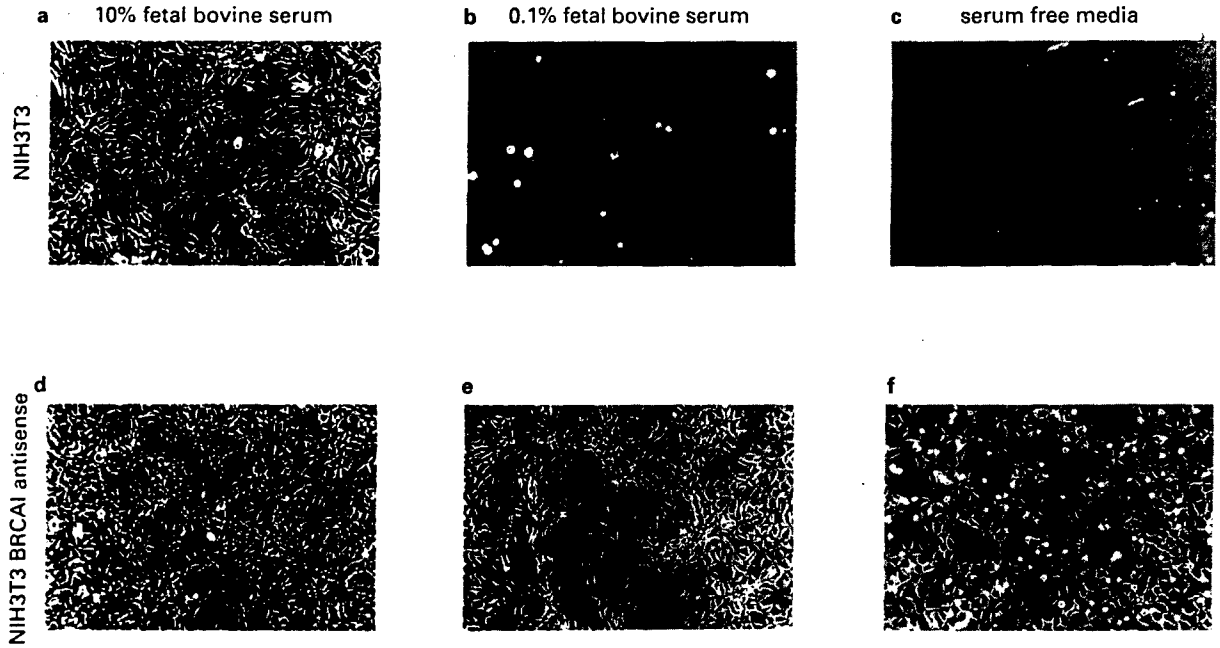


Figure 3 Growth properties and morphology of BRCA^{AS} transfectants. Phase-contrast photomicrographs of NIH3T3 (a-c) and BRCA^{AS} cell lines (d-f). Cells were cultured in normal media (10% FBS/DMEM) (a and d); low serum (0.1% FBS/DMEM) (b and e); serum free media (DMEM alone) (c and f)

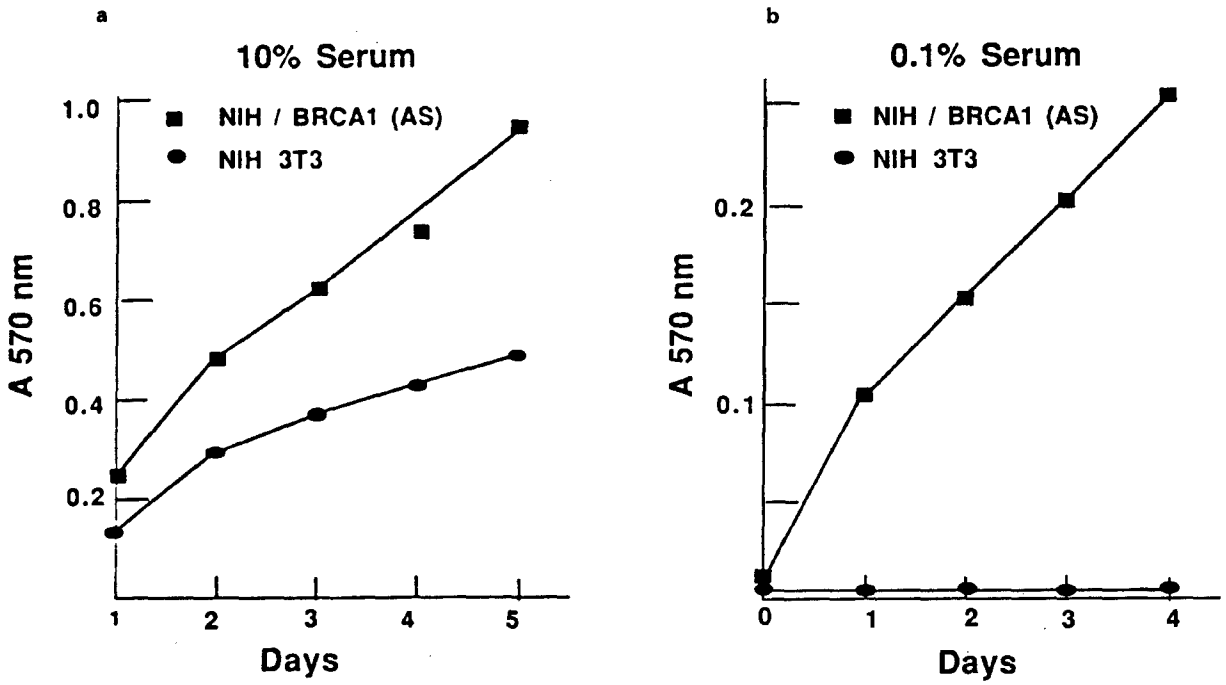


Figure 4 Inhibition of BRCA1 expression by antisense RNA accelerates NIH3T3 cell proliferation. Growth curves represent numbers of viable cells as measured by MTT dye assay (Loveland *et al.*, 1992) after different periods of cultivation in medium containing 10% FBS (a) or in 0.1% serum (b). The points represent mean of duplicates from a representative experiment

In summary, our results demonstrate that the BRCA1 gene product is a nuclear phosphoprotein which has tumor-suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 results in neoplastic transformation. Our study provides the first direct biological evidence for the role of BRCA1 in transformation. The mechanism by which BRCA1 regulates cell proliferation is not known. The cell lines generated in this study should be useful to study the molecular mechanism involved in the function of BRCA1 gene. Knowledge of the pathways from inhibition of BRCA1 function leading to the progression to cancer will be important for the development of diagnostic kits and for designing targeted therapeutic strategies. Future efforts will be directed towards directly testing the BRCA1 gene product for growth inhibitory function.

Materials and methods

Plasmid construction and transfections

cDNA of BRCA1 (V Rao and ESP Reddy, unpublished results) containing the coding exons were subcloned into pcDNA vector by PCR using the published BRCA1 primers (Miki *et al.*, 1994). Purified DNA (15 μg) of pcDNA expression vector or vector containing the sense/antisense BRCA1 cDNAs were transfected into mouse NIH3T3 cells using the Strategene kit according to the manufacturers instructions. G418 resistant colonies were picked and propagated in the selective medium.

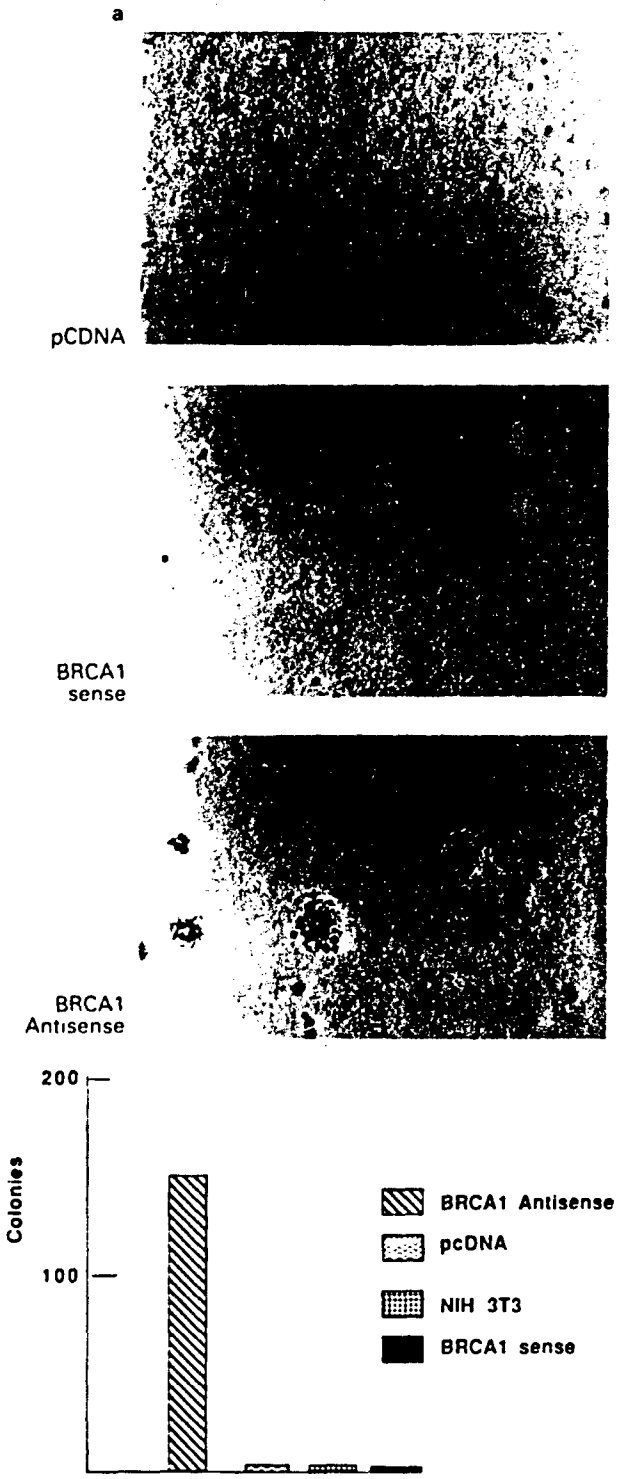


Figure 5 Growth of BRCA1^{AS} cells in soft agar (**a**) 2×10^3 cells per dish of pcDNA vector transfectant, BRCA1^S transfectant and BRCA1^{AS} transfectant were analysed for anchorage independent growth. (**b**) clonogenicity of BRCA1^{AS} cells; pcDNA vector cells; parental NIH3T3, and BRCA1^S cells

Western blot analysis

Cell extracts were prepared from exponentially growing cells, the protein concentration in the extracts were determined by Bradfords method (Bio Rad) and $\approx$18–87 μg of protein from each of the extracts were loaded on a 10% SDS–PAGE as described previously (Rao *et al.*, 1989). After electrotransfer onto PVDF membrane, the filter was probed with a polyclonal BRCA1 peptide antibody and visualized using a chemiluminiscent assay as described by the manufacturer (Clontech kit) and exposed to Kodak X-AR film. Duplicate SDS–PAGE gels were run for each experiment and stained with coomassie blue.

MTT dye assay

The MTT metabolic assay was performed as described (Loveland *et al.*, 1992). In brief, cells were seeded at a density of 1×10^4 cells into microtitre plates and incubated in 10% FBS or 0.1% FBS media. At five 24 h intervals viable cells were stained for 4 h with the MTT dye and absorbance was read at 570 nm. The experiments were performed in duplicates or triplicates and reproduced at least three times.

Anchorage independence assay

Soft agar growth assay was done in 0.3% agar/DMEM/10% FBS and plated on a base of 0.5% agar/DMEM/10% FBS. Cells were plated at a concentration of 2×10^3 cells per 35 mm plate in soft agar containing DMEM and 10% FBS. Colonies greater than 100 μm in diameter were scored after 3 weeks. Each soft agar assay was performed in triplicates.

Immunohistochemistry

MCF7 and Rat1 cells cultured in chamber slides were washed in PBS, fixed with 3.7% formaldehyde in PBS at room temperature for 30 min. This was followed by washes in PBS and 30 min block in blocking serum (VECTASTAIN, ABC system from Vector). The specimens were incubated with primary BRCA1 carboxyterminal peptide antibody diluted 1:100 at room temperature for 2 h. After washing with PBS, slides were incubated for an hour with diluted biotinylated secondary antibody solution. The slides were washed with PBS and incubated for 30 min with Vectastain ABC reagent. Slides were further washed and incubated for 5 min in 0.01% H_2O_2–0.05% DAB solution. Slides were washed for 5 min in water, mounted in cytoseal 60 (Stephens scientific) and photographed on a immunofluorescence microscope (Olympus).

Immunoprecipitation

Briefly, confluent 100 mm plates of HL60 cells were labelled with [^{35}S] trans label for 3 h. The cells were lysed in radio immunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti-BRCA1 peptide antibody or preimmune serum as described previously (Rao and Reddy, 1993). The samples were subjected to 12% SDS polyacrylamide gel electrophoresis, fluorography and autoradiography.

Acknowledgements
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Induction of apoptosis by the tumor suppressor protein BRCA1

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The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor. To investigate the role of BRCA1 in apoptosis, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1. The expression of BRCA1 protein in the BRCA1 transfectants were analysed by immunofluorescence and immunohistochemistry. The BRCA1 transfectants showed a flattened morphology compared to the parental cells. We show that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death. These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis. Thus, since a wide variety of human malignancies like breast and ovarian cancers have a decreased ability to undergo apoptosis, this could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

Keywords: BRCA1 proteins; tumor suppressor; breast and ovarian cancers; apoptosis; therapy

Introduction

Breast cancer is one of the most common malignancy affecting women in the United States and ovarian cancer although less frequent than breast cancer is the fourth leading cause of cancer mortality among women. The breast and ovarian cancer susceptibility gene BRCA1, was recently isolated and the conceptual cDNA encodes a 1863 aminoacid protein with an amino-terminal Zinc finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994). We and others have identified multiple BRCA1 proteins approximately 185–220, 160, 145, 100, 52 and 38 kD in both human and mouse cells (Rao *et al.*, 1996; Chen *et al.*, 1995). It remains to be seen whether these proteins represent isoforms of BRCA1 or its related proteins. Recently, we like others have identified alternatively spliced transcripts of the BRCA1 gene (Rao, unpublished results). A recent report indicated that the BRCA1 gene product is localized in the nucleus of several normal cell lines including breast and tumor cells other than breast and ovary (Chen *et al.*, 1995). They have detected BRCA1 mainly in the cytoplasm of almost all breast and ovarian cancer cell lines examined. These results suggested aberrant subcellular localization of BRCA1 in breast cancer (Chen *et al.*, 1995). Interestingly, our results show BRCA1 to be localized mainly in the nucleus (or perinuclear) or cytoplasm or both of several normal or cancer cells (Rao, unpublished results) indicating variable subcellular localization of the BRCA1 proteins. Our results suggest that the subcellular localization of BRCA1 may be determined by the cell cycle status of the cells (Rao, unpublished results). Our results show that BRCA1 interacts with cyclin dependent kinases suggesting a role for BRCA1 in cell cycle regulation (Rao, unpublished results). We have previously reported that the BRCA1 gene product to be a nuclear protein with tumor suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 resulted in neoplastic transformation (Rao *et al.*, 1996). We have recently observed that introduction of variant BRCA1 gene into human cancer cells results in suppression of growth and neoplastic phenotype (Rao *et al.*, unpublished results) implicating a direct role for BRCA1 in growth and tumor suppression.

Results and discussion

To study the function of BRCA1 genes in the regulation of apoptosis, we have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1. These BRCA1^s cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis (Figure 1a) and immunoperoxidase staining (Figure 1a) using BRCA1 polyclonal antibody as described previously (Rao *et al.*, 1996). The nuclear and cytoplasmic staining was brighter and stronger in BRCA1^s transfectants compared to parental NIH3T3 cells (Figure 1a). The morphology of the BRCA1^s transfectants were different from that of the parental NIH3T3 cells. The BRCA1^s cells are shorter and flatter when compared to the parental NIH3T3 cells (Figure 1b) and with several weeks in continuous culture the BRCA1^s cells become spindle shaped with elongated processes leading to their detachment. These results suggest that constitutive high level expression of the BRCA1 gene product for a prolonged period of time may result in apoptosis.

One of the most efficient ways of triggering the apoptotic response in fibroblasts is the removal of serum (Jimenez *et al.*, 1995). Thus we next investigated the effect of serum withdrawal on the induction of apoptosis in the BRCA1^s transfectants and compared it with that of the NIH3T3 cells. Subconfluent NIH3T3 and BRCA1^s cells were grown in medium containing

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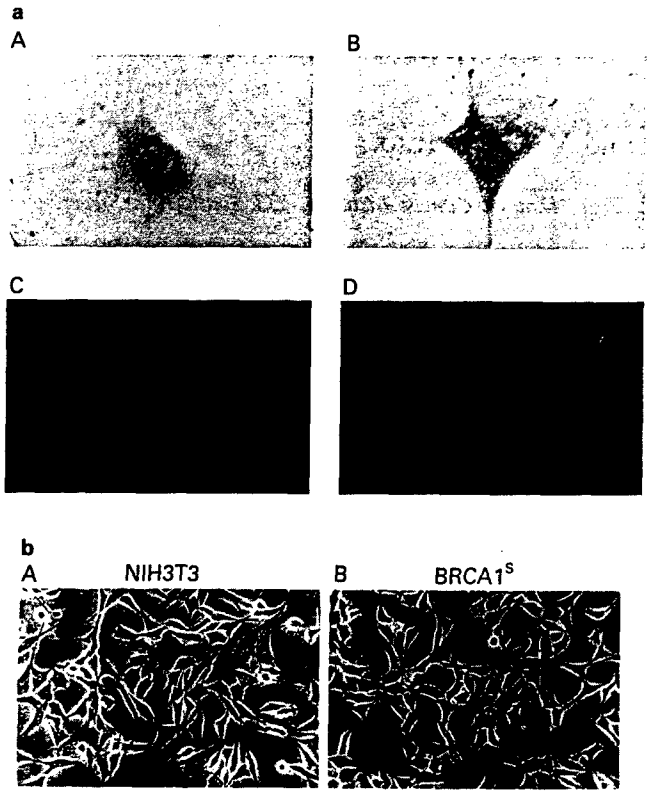


Figure 1 Detection of BRCA1 protein(s) in NIH3T3 and BRCA1^s cells by immunohistochemically and immunofluorescence analysis. (**a**) Immunoperoxidase (A, B) and immunofluorescence (C, D) analysis. A. NIH3T3; B, BRCA1^s; C, NIH3T3; D, BRCA1^s. (**b**), Morphology of the BRCA1^s transfectants. Phase-contrast photomicrographs of NIH3T3 (A) and BRCA1^s cell lines cultured in normal media (10% FBS/DMEM)

either 10% or 0% fetal bovine serum (FBS). After 24 h the cells were collected and subjected to flow cytometric analysis. The BRCA1^s transfected cells showed enhanced rates of apoptosis under serum depleted conditions, as measured by the appearance of an additional 'sub G1' peak on flow cytometry (Figure 2a). This peak is associated with high levels of DNA degradation. High levels of apoptotic cells in the sub G_0/G1 peak (A_p) population were measured in BRCA1^s cells (A_p value 75%) whereas the control cell line NIH3T3 showed lower levels of apoptosis under identical conditions (Figure 2a). BRCA1^s cells did not show any significant apoptosis levels when grown under normal conditions of DMEM supplemented with 10% FBS (Figure 2a).

The above results of apoptosis in BRCA1^s transfectants was further comfirmed by DNA fragmentation assay. Here NIH3T3 and BRCA1^s transfectant cells were cultured in serum free media for 24, 48 and 72 h and then analysed for DNA fragmentation in agarose gels. The BRCA1^s transfected cell line showed the production of a typical oligonucleosomal DNA ladder (Figure 2b; B, lanes 1–3) indicating activation of apoptosis. This effect was readily seen for BRCA1^s cell lines cultured in serum free media but not for the parental NIH3T3 cells grown under identical conditions (Figure 2b; A, lanes 1 to 3). These results indicate that over expression of BRCA1 accelerates apoptosis in serum depleted NIH3T3 cells.

Apoptosis can be induced by calcium ionophore, A23187, in thymocytes (McConkey *et al.*, 1989). This led us to examine apoptosis in BRCA1^s cells during A23187 treatment. NIH3T3 and BRCA1^s cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method (Bendall *et al.*, 1994). Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M of the cell cycle were evaluated by computer analysis using EPICS profile analyzer. The BRCA1^s transfected cells showed accelerated rates of apoptosis (A_p value 82%) in presence of calcium ionophore (Figure 3a). Measurement of apoptosis through the sub G1 peak in the DNA histogram has the advantage of simplicity. But it has the disadvantage that since all the cells are fixed there is no distribution between viable and dead cells. Therefore the viability of both NIH3T3 cells and BRCA1^s cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that majority of the BRCA1^s cells treated with calcium ionophore A23187 were dead whereas most of the control NIH3T3 cells survived (Figure 3). These results suggest that BRCA1 induces death in NIH3T3 cells.

Apoptosis in the BRCA1^s transfectants was further confirmed in two ways. First, NIH3T3 and BRCA1^s cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hochest 33258 (Oberhammer *et al.*, 1994). Majority of the nuclei of BRCA1^s showed strong chromatin condensation and nuclear degradation into small, spherial nuclear particles of condensed chromatin characteristic of apoptosis (Figure 3c), whereas the parental NIH3T3 cells did not show any significant change in the staining pattern (Figure 3). Second, the analysis of DNA degradation upon treatment of BRCA1^s transfectants with calcium ionophore confirmed induction of apoptosis. Figure 3d shows that the DNA of BRCA1^s cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder whereas the parental NIH3T3 cells had no significant DNA degradation (Figure 3d). These results suggested that calcium ionophore induces apoptosis more readily in BRCA1^s cells than NIH3T3 cells. All the above results shown for one clone of NIH3T3–BRCA1^s cells, have been reproducibly obtained with several other independent clonal isolates of NIH3T3–BRCA1^s cell lines (data not shown).

In an attempt to understand the role of BRCA1 genes in the regulation of apoptosis of human breast cancer cells, we have transfected MCF7 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1 (Rao *et al.*, unpublished results). These BRCA1^s cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis, immunoperoxidase staining and Western blot analysis (Rao *et al.*, unpublished results). Apoptosis in the MCF-7 BRCA1^s transfectants were analysed after treatment with calcium ionophore A23187. MCF-7 and BRCA1^s cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by Flow cytometry with propidium iodide staining. The BRCA1^s transfected MCF-7 cells showed accelerated

rates of apoptosis (A_p value 75%) in the presence of calcium ionophore (Figure 4a). The viability of both MCF-7 cells and BRCA1^s cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 BRCA1^s cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived (Figure 4b). These results suggest that BRCA1 induces death in MCF-7 cells. The induction of apoptosis in the BRCA1^s transfectants was further confirmed by analysis of DNA fragmentation upon treatment of BRCA1^s transfectants with calcium ionophore. The DNA of BRCA1^s cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (Figure 4c). These results suggest that over expression of BRCA1 in breast cancer cells results in apoptosis.

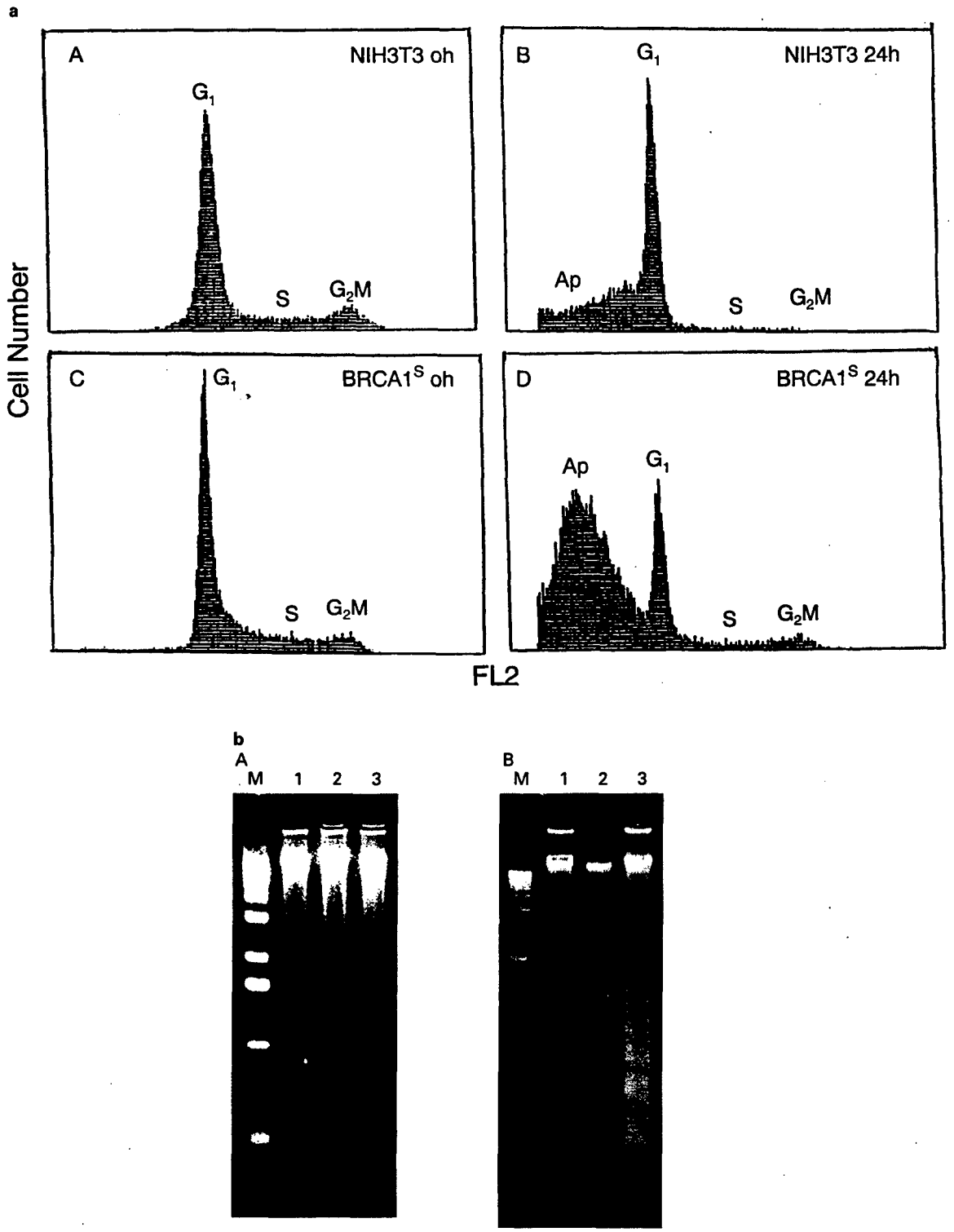


Figure 2 BRCA1 over expression induces apoptosis in serum depleted NIH3T3 cells. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by serum deprivation. A, NIH3T3, 0 h; B, NIH3T3, 24 h; C, BRCA1^s 0 h; D, BRCA1^s 24 h. (**b**) Over expression of BRCA1 gene induces DNA fragmentation typical of apoptosis in serum deprived cells. A, control NIH3T3 cells serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3) and B, BRCA1^s cell serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3)

In summary, our results demonstrate that the BRCA1 gene product which is a nuclear phosphoprotein (Rao *et al.*, 1996; Chen *et al.*, 1995) with tumor suppressor properties (Rao *et al.*, 1996; Rao *et al.*, unpublished results) functions as an inducer of apoptosis similar to rho, c-myc, p53, E1A and rel (Jimenez *et al.*, 1995; Fisher, 1994; Hoffman and Libermann, 1994; Yonish-Rouach *et al.*, 1991). The BRCA1 cDNA used in this study lacks majority of exon 11, suggesting that this region of exon 11 is dispensable for the apoptotic function of BRCA1. The precise mechanism by which BRCA1 triggers cell death remains to be investigated. It may be possible that BRCA1 gene products function as transcriptional regulators that may either activate death inducing genes or repress death inhibiting genes leading to apoptosis. Alternatively, BRCA1 may activate apoptosis inducing

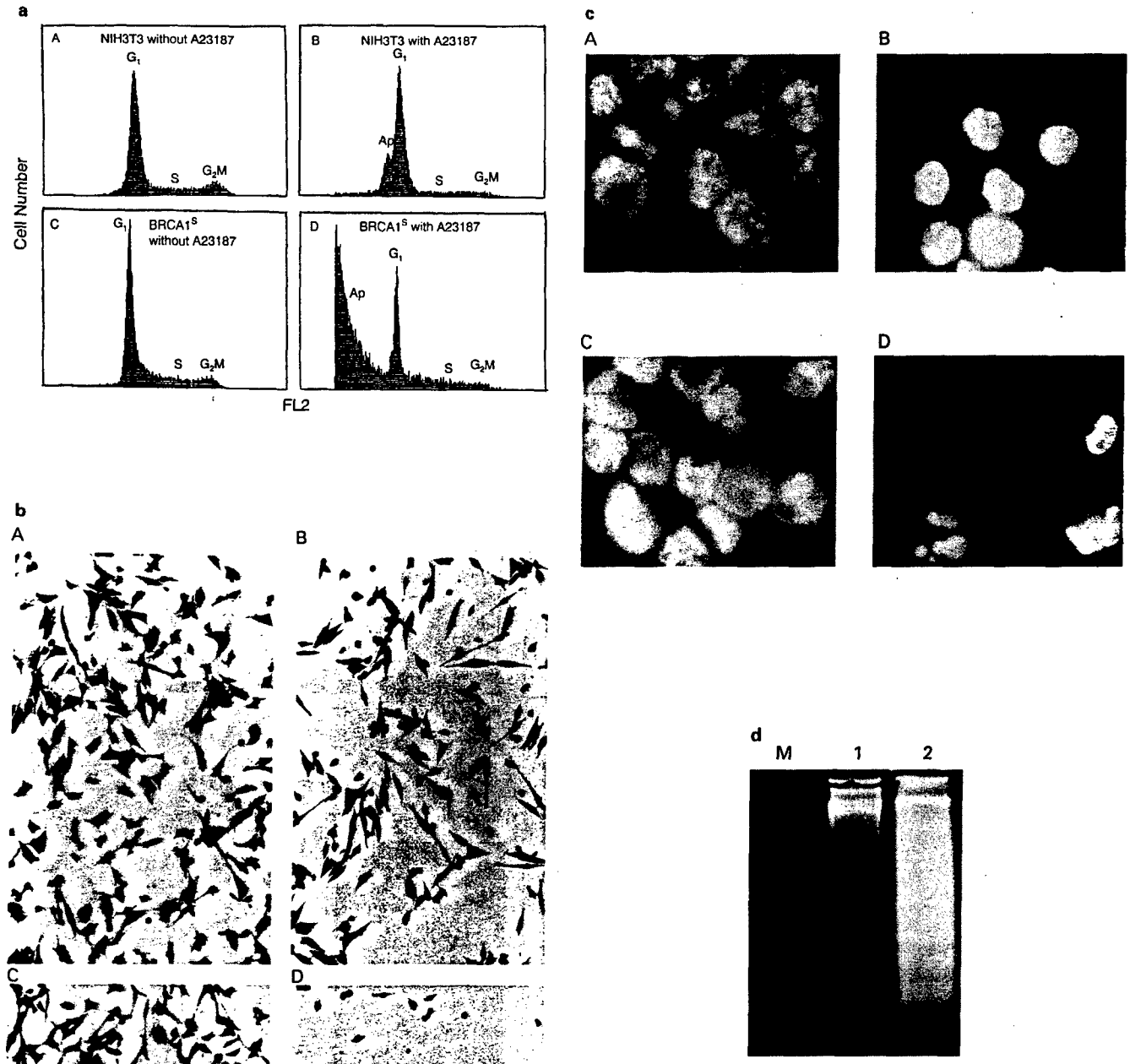
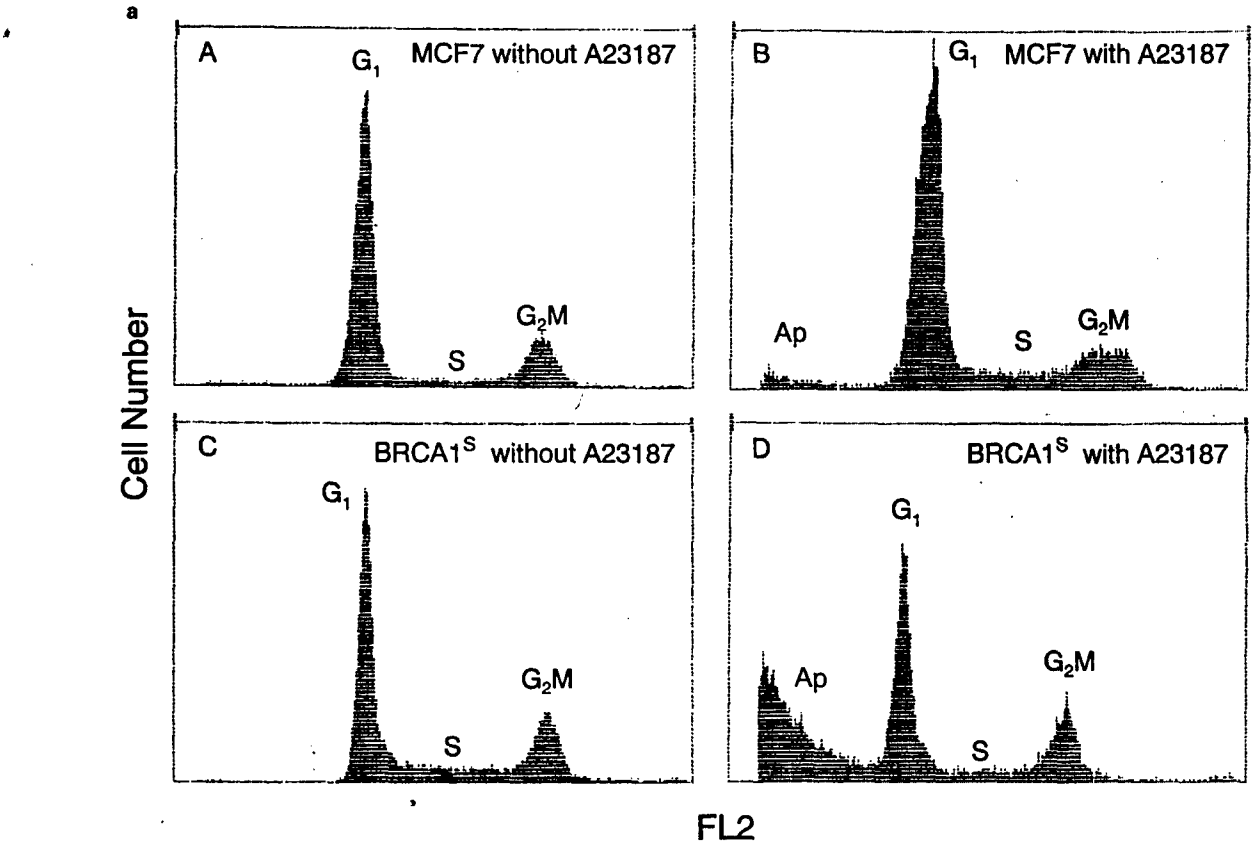
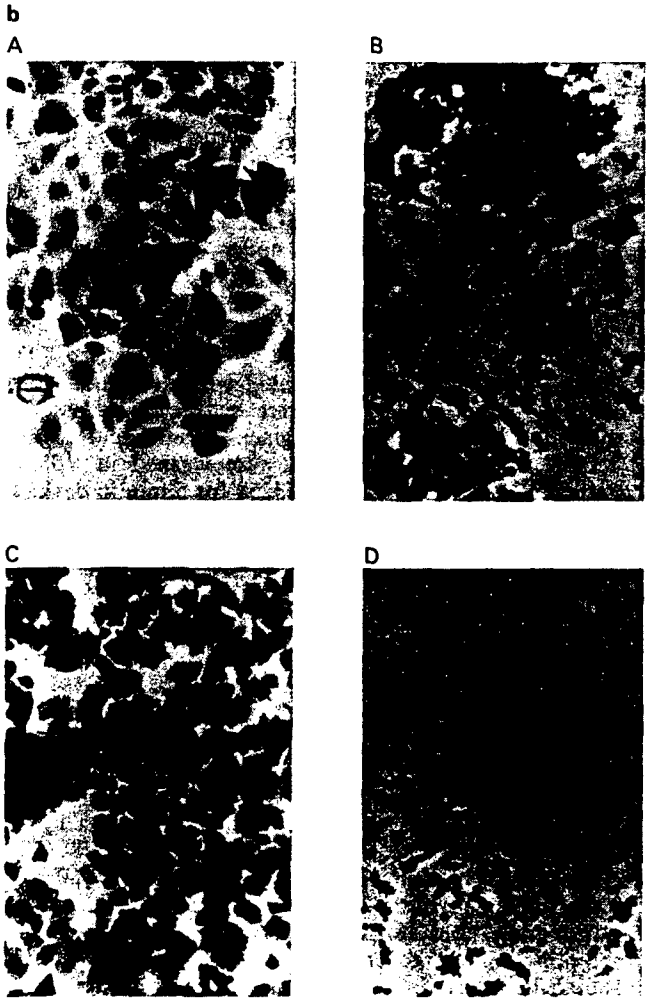


Figure 3 BRCA1 induces apoptosis in NIH3T3 cells after calcium ionophore treatment. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (**b**) Viability of NIH3T3 and BRCA1^s cells treated with and without calcium ionophore for 24 h. A, NIH3T3: B, NIH3T3 treated with calcium ionophore; C, BRCA1^s; D. BRCA1^s treated with calcium ionophore. (**c**) Chromatin condensation shown morphologically by Hoeschst staining of the BRCA1^s cell line induced to undergo apoptosis by calcium ionophore treatment for 24 h. A, NIH3T3 in the absence of calcium ionophore; B, NIH3T3 in the presence of calcium ionophore; C, BRCA1^s in the absence of calcium ionophore; D. BRCA^s in the presence of calcium ionophore. (**d**) DNA fragmentation induced by BRCA1 overexpression. 1, NIH3T3 treated with calcium ionophore; 2, BRCA1^s treated with calcium ionophore





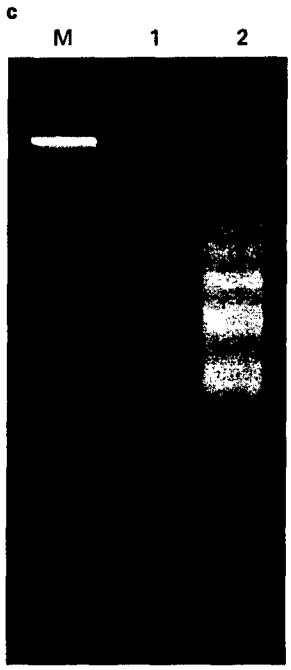


Figure 4 BRCA1 over expression induces apoptosis in MCF-7 cells after calcium ionophore treatment. **(a)** Flow cytometry analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. **(b)** Cell viability of MCF-7 and BRCA1^{s} cells treated with and without calcium ionophore for 24 h. A, MCF-7; B, MCF-7 cells treated with calcium ionophore; C, BRCA1^{s}; D, BRCA1^{s} treated with calcium ionophore. **(c)** DNA fragmentation induced by BRCA1 over-expression. Lane 1, MCF-7 cells treated with calcium ionophore; 2, BRCA1^{s} cells treated with calcium ionophore

proteins or target apoptosis inhibiting proteins through direct protein-protein interactions. In the mouse mammary gland BRCA1 expression was found to be elevated during pregnancy following treatment with ovarian hormones (Lane *et al.*, 1995; Marquis *et al.*, 1995) and in human breast cancer cells BRCA1 mRNA levels were found to be regulated by steroid hormone estrogen and progesterone (Gudas *et al.*, 1995). Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis (Thompson, 1995). Our results suggest that lack or decreased levels of expression of functional BRCA1 gene product in breast and ovarian cancers may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis for the treatment of breast and ovarian cancers.

Materials and methods

Cell lines

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FCS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. MCF-7 cells were transfected with BRCA1 cDNA as described previsouly (Rao *et al.*, 1996; Rao *et al.*, unpublished results).

Immunohistochemistry

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. NIH3T3 and BRCA1^s transfectant cells were cultured in chamber slides and processed for immunohistochemistry using the BRCA1 peptide antibody as described previously (Rao *et al.*, 1996).

Immunofluorescence

Immunofluorescence analysis was done as described previously (Dyck *et al.*, 1994). In brief, NIH3T3 and BRCA^s cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, following by washing with PBS and blocking in blocking solution (3% bovine serum albumin-0.5% Triton X-100 in PBS) for 30 min at room temperature. The cells were incubated with primary BRCA1 peptide antibody diluted 1:100 for 2 h at room temperature, then washed three times with PBS and were incubated with the secondary antibody (FITC conjugated goat anti rabbit IgG (Cappel). After rinsing in PBS, the slides were mounted with fluoresecence mounting media (Vector) and photographed on an immunofluorescence microscope.

Flow cytometry analysis

Subconfluent to confluent NIH3T3, BRCA1^s cells were incubated in 10% FBS or 0% FBS media. After 24 h both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at −18°C overnight. Cells were pooled, washed in PBS and stained with propidium iodide (20 μg/ml) and incubate with 20 μg/ml of RNAse A at 4°C in the dark overnight. Samples were analysed using a EPICs profile analyzer. Histograms showing the total DNA content at FL2 vs cell number are shown.

Flow cytometry analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done similar to that described in Figure 2 legend except for the treatment with 20 μM calcium ionophore A 23187 (Sigma) for 24 h.

DNA fragmentation

DNA fragmentation assay was done as described (Kondo *et al.*, 1995). In brief, confluent NIH3T3 and BRCA1^s cells grown in DMEM supplemented with 10% FBS and penicillin streptomycin were changed into media containing 0% FBS for 24, 48 and 72 h. After the indicated incubation periods, both adherent and detached cells were collected ($2-5\times10^6$ cells), washed once in TBS buffer and lysed in 1 ml of 100 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS and 20 μg/ml RNAse A (pH 8.0) and incubated at 37°C for 30 min. Proteinase K at a final concentration of 100 μg/ml was added and further incubated for 3 h at 55°C. After extraction with an equal volume of phenol:chloroform: isoamyl alcohol, followed by re-extraction with phenol:chloroform: isoamyl alcohol. The DNA was precipitated from the aqueous phase with sodium acetate and two volumes of ethanol, the DNA pellet was dissolved in TE buffer and analysed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

DNA fragmentation analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done as described in Figure 2 legend except for the treatment of A23187 (20 μM) for 24 h before analysis on a 2% agarose gel.

Cell viability

Subconfluent NIH3T3, MCF-7-BRCA1^s cells seeded into six well plates and grown in DMEM 10% FBS were treated with 20 μM calcium ionophore A23187 for 24 h. After which cells were washed with PBS and fixed in methanol:glacial acetic (3:1). Cells were stained with crystal violet. After washing in water, the cells were visualized and photographed under an Olympus microscope at 200× magnification.

Chromatin condensation

NIH3T3 and BRCA1 cells treated with or without 20 μM calcium ionophore for 24 h were cultured on glass cover slips and fixed in methanol:glacial acetic acid (3:1) at −18°C for 30 min. The cells were washed in PBS and stained with 8 μg/ml Hochest 33258 for 5 min in dark. The coverslips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope.

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Wet thank G Chipitsyna for excellent technical help. We thank other colleagues of Rao and Reddy laboratories for their cooperation. This work is supported in part by NIH grants CA57322 to VNR and NIH grant CA 58642 and ACS grant VM#69 to ESPR and DAMD17-94-4280 by US Army medical research (VNR). The contents of the information does not necessarily reflect the position or the policy of the government and no official endorsement should be inferred.

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BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases

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BRCA1, a familial breast and ovarian cancer susceptibility gene encodes nuclear phosphoproteins that function as tumor suppressors in human breast cancer cells. Previously, we have shown that overexpression of a BRCA1 splice variant BRCA1a accelerates apoptosis in human breast cancer cells. In an attempt to determine whether the subcellular localization of BRCA1 is cell cycle regulated, we have studied the subcellular distribution of BRCA1 in asynchronous and growth arrested normal, breast and ovarian cancer cells using different BRCA1 antibodies by immunofluorescence and immunohistochemical staining. Upon serum starvation of NIH3T3, some breast and ovarian cancer cells, most of the BRCA1 protein redistributed to the nucleus revealing a new type of regulation that may modulate the activity of BRCA1 gene. We have also characterized two new variant BRCA1 proteins (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine. Immunofluorescence and Western blotting analysis indicate cytoplasmic and nuclear localization of BRCA1a and BRCA1b proteins. To elucidate the biological function of BRCA1, we created a bacterial fusion protein of glutathione-transferase (GST) and BRCA1 zinc finger domain and detected two cellular proteins with molecular weights of approximately 32 and 65 kD, one of which contains phosphotyrosine designated p32 and p65 BRCA1 interacting proteins (BIP) that specifically interact with BRCA1. Western blot analysis of BIP with cyclins/CDKs and E2F antisera indicated association with cdc2, cdk2, cdk4, cyclin B, cyclin D, cyclin A and E2F-4 but not with cdk3, cdk5, cdk6, E2F-1, E2F-2, E2F-3, E2F-5 and cyclin E. Furthermore, we have also demonstrated a direct interaction of *in vitro* translated BRCA1a and BRCA1b proteins with recombinant cyclin A, cyclin B1, cyclin D1, cdc2, cdk2 and E2F fusion proteins *in vitro*. Taken together these results seem to suggest that BRCA1 could be an important negative regulator of cell cycle that functions through interaction with E2F transcriptional factors and phosphorylation by cyclins/cdk complexes with the zinc ring finger functioning as a major protein-protein interaction domain. If the interactions we observe *in vitro* is also seen *in vivo* then it may be possible that lack or impaired binding of the disrupted BRCA1 proteins to E2F, cyclins/CDKs in patients with mutations in the zinc finger domain could deprive the cell of an important mechanism for braking cell proliferation leading to the development of breast and ovarian cancers.

Keywords: BRCA1a; BRCA1b; zinc finger; cyclins; CDKs; E2F

Introduction

Mutations in the breast and ovarian cancer susceptibility gene BRCA1, accounts for half of the inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995) and only 10% of the sporadic ovarian cancers (Futreal *et al.*, 1994; Hosking *et al.*, 1995; Merajver *et al.*, 1995). The BRCA1 cDNA codes for a 1863 amino acid protein with an amino terminal zinc ring finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994) typical of several transcriptional factors. Recently, the C terminal region of BRCA1 was shown to activate transcription in a heterologous GAL-4 system (Chapman and Verma, 1996; Monteiro, 1996; Rao *et al.*, unpublished results). Several groups have cloned and studied the developmental patterns of expression of murine BRCA1 (Lane *et al.*, 1995; Marquis *et al.*, 1995; Abel *et al.*, 1995; Sharan *et al.*, 1995). Expression was found to be high in rapidly proliferating tissues (Lane *et al.*, 1995; Marquis *et al.*, 1995) particularly those undergoing differentiation suggesting a role for BRCA1 in cellular growth and differentiation. We and others have shown the BRCA1 gene product to be a nuclear phosphoprotein (Chen *et al.*, 1995; Rao *et al.*, 1996; Scully *et al.*, 1996), that when over expressed in breast and ovarian cancer cells results in growth inhibition *in vitro* and *in vivo* in breast cancer cells (Holt *et al.*, 1996; Rao *et al.*, unpublished results). Conversely, inhibition of BRCA1 expression by antisense RNA in mouse fibroblasts or by antisense oligonucleotides in breast cancer cells resulted in transformation of mouse fibroblasts as well as increased the rate of growth of breast cancer cells (Thompson *et al.*, 1995; Rao *et al.*, 1996). We have recently reported a new function for the BRCA1 splice variant BRCA1a in the regulation of apoptosis of human breast cancer cells (Shao *et al.*, 1996). Previously, the BRCA1 gene product was shown to be localized in the nucleus (Chen *et al.*, 1995; Rao *et al.*, 1996). Since then there have been several

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differences in the literature regarding the size and subcellular location of BRCA1 (Chen *et al.*, 1995, 1996; Jensen *et al.*, 1996; Scully *et al.*, 1996; Thakur *et al.*, 1997; Wilson *et al.*, 1997). Two proteins BARD1 and Rad51, a human homolog of bacterial Rec A were shown to interact both *in vitro* and *in vivo* with BRCA1 indicating a role for BARD1 in tumor suppression of BRCA1 and a role for BRCA1 in the control of recombination and genomic integrity (Wu *et al.*, 1996; Scully *et al.*, 1977). In the present study, we have further investigated the subcellular localization of BRCA1 protein using several BRCA1 specific antibodies in normal, breast and ovarian cancer cells grown under different serum culture conditions and conclude that the BRCA1 proteins are nuclear phosphoproteins that are transported to the nucleus in the absence of serum. Our results suggest that BRCA1a and BRCA1b, two BRCA1 splice variants that are localized mainly in the cytoplasm with a small fraction going into the nucleus, are phosphoproteins containing phosphotyrosine that associate via their amino-terminal zinc ring finger domain with E2F transcriptional factors, cyclins/cdk complexes suggesting a function for this domain in mediating protein-protein interaction and a role for BRCA1 in cell cycle regulation.

Results

Subcellular localization of BRCA1 proteins

In an attempt to understand the normal function of the BRCA1 protein, we have generated polyclonal antibodies against different regions of the human BRCA1 protein. Initially, we have studied the subcellular distribution of BRCA1 in normal human mammary epithelial cell Hs578 Bst and several human breast cancer cell lines HBL-100, CAL-51, MDA MB-453, T-47D, BT-474, CAMA-1 and ZR 75-1 by immunohistochemistry and immunofluorescence staining. BRCA1 was detected mainly in the cytoplasm with weak nuclear staining of normal human breast epithelial cell Hs578 Bst and several breast tumor cell lines HBL-100, MDA MB-453, T-47D, BT474, CAMA-1 and ZR 75-1 (Figure 1a). One cell line CAL-51, which was originally obtained fro a patient with invasive adenocarcinoma with extensive intraductal involvement (Gioanni *et al.*, 1990), contained three distinct populations of cells, some in which BRCA1 was localized in the cytoplasm, some in the perinucleus and some in the nucleus (Figure 1a). We next studied the subcellular distribution of BRCA1 in two ovarian carcinoma cell lines NIH:OVCAR-3 and SK-OV-3. In NIH:OVCAR-3 cells BRCA1 was localized mainly to the cytoplasm (Figure 1b) and in SK-OV-3 BRCA1 was localized mainly in the nucleus (Figure 1b). We also studied the subcellular distribution of BRCA1 in several normal and tumor cells. BRCA1 was found to be distributed mainly in the cytoplasm of NIH3T3 mouse fibroblast cells and Saos-2 cells (Figure 1c), both in the cytoplasm and nucleus of BRACA1a transfected NIH3T3 cells, HeLa, Colo 320, A431 and PC12 cells (Figure 1c) and mainly in the nucleus with weak cytoplasmic staining of BALB/3T3 cells (Figure 1c). All these results suggested variable, subcellular distribution of BRCA1 proteins. These results were obtained using different BRCA1 antibodies.

Subcellular localization and tyrosine-phosphorylation of BRCA1a and BRCA1b proteins

We have next studied the *in vivo* phosphorylation of BRCA1 proteins in HL60 cells. Immunoprecipitation of lysates from HL60 cells labeled with ^{32}P-phosphoric acid using BRCA1 polyclonal antibody revealed three major bands with molecular weights of $\approx$175, 125 and 110 kD (Figure 2a). All these results suggest BRCA1 to be a phosphoprotein. In order to explain the differences in the subcellular localization of BRCA1, we speculated whether this could be due to the presence of multiple splice variants wherein some could be preferentially sequestered in the cytoplasm and some could translocate to the nucleus as observed recently by other investigators (Thakur *et al.*, 1977; Wilson *et al.*, 1977). So, we subcloned the differentially spliced human BRCA1 cDNA, BRCA1a into pFLAG-CMV-2 expression vector (Eastman Kodak Company) which contains a FLAG epitope-tag sequence at the N-terminal and this tag allows detection of BRCA1a protein with the use of FLAG antibody. When transfected into COS cells the FLAG BRCA1a protein was found to be localized mainly in the cytoplasm with weak nuclear staining (Figure 2b). Subcellular fractionation of the transfected COS cells into total and nuclear fractions followed by Western blot analysis using the same FLAG antibody revealed two polypeptides migrating at 105–110 kD mainly in the cytoplasm with a small fraction of the total BRCA1a protein in the nuclear fractions, which agrees with our immunofluorescence data. These results suggest BRCA1a p110 to be a localized both in the cytoplasm and nucleus, the doublet protein bands observed could represent hyper and hypophosphorylated forms of BRCA1a proteins, similar to the 220 kDa BRCA1 protein (Chen *et al.*, 1996a). Western blot analysis of FLAG immunoprecipitates obtained from p-FLAG-CMV-2-BRCA1a transfected COS cells using phosphotyrosine antibodies revealed BRCA1a to be a phosphoprotein containing tyrosine (Figure 2c lane 3) which migrated with mobility similar to an endogenous BRCA1 tyrosine phosphorylated band seen in HL60 cells (Figure 2c). All these results suggest that BRCA1a is an $\approx$110 kD phosphoprotein which contains tyrosine. Similarly, BRCA1b was also found to be a phosphoprotein phosphorylated on tyrosine migrating with a molecular weight of $\approx$100 kD (data not shown). We next studied the subcellular distribution of FLAG-BRCA1a in breast cancer cell line CAL-51 after transfection of pFLAG-CMV-2-BRCA1a plasmid into these cells followed by immunofluorescence analysis. Here, we observed BRCA1a protein to be distributed both in the cytoplasm as well as in the nucleus of serum fed cells, the cytoplasmic staining appeared to be much stronger compared to the weak nuclear staining (Figure 2d).

Intracellular localization of BRCA1 proteins in serum-grown and serum deprived mouse fibroblasts

Our results suggested that the interesting differences in the subcellular localization of BRCA1 observed by us

and others may not be due to using different BRCA1 antibodies or aberrant localization of BRCA1 in breast tumor cells. We therefore speculated whether this could be due to culturing the cells in different serum culture conditions. In order to test the hypothesis whether the nuclear localization of BRCA1 is dependent on the proliferation state of the cell, we studied the subcellular distribution of BRCA1 in asynchronous and serum deprived NIH3T3 cells using different BRCA1-specific antibodies developed by us by immunofluorescence and immunohistochemical methods. In asynchronous serum fed NIH3T3 cells BRCA1 was found to be localized predominantly in the cytoplasm with weak nuclear staining (Figure 3a). In contrast, in serum starved quiescent cells most of the BRCA1 was found to be localized in the nucleus with little cytoplasmic staining.

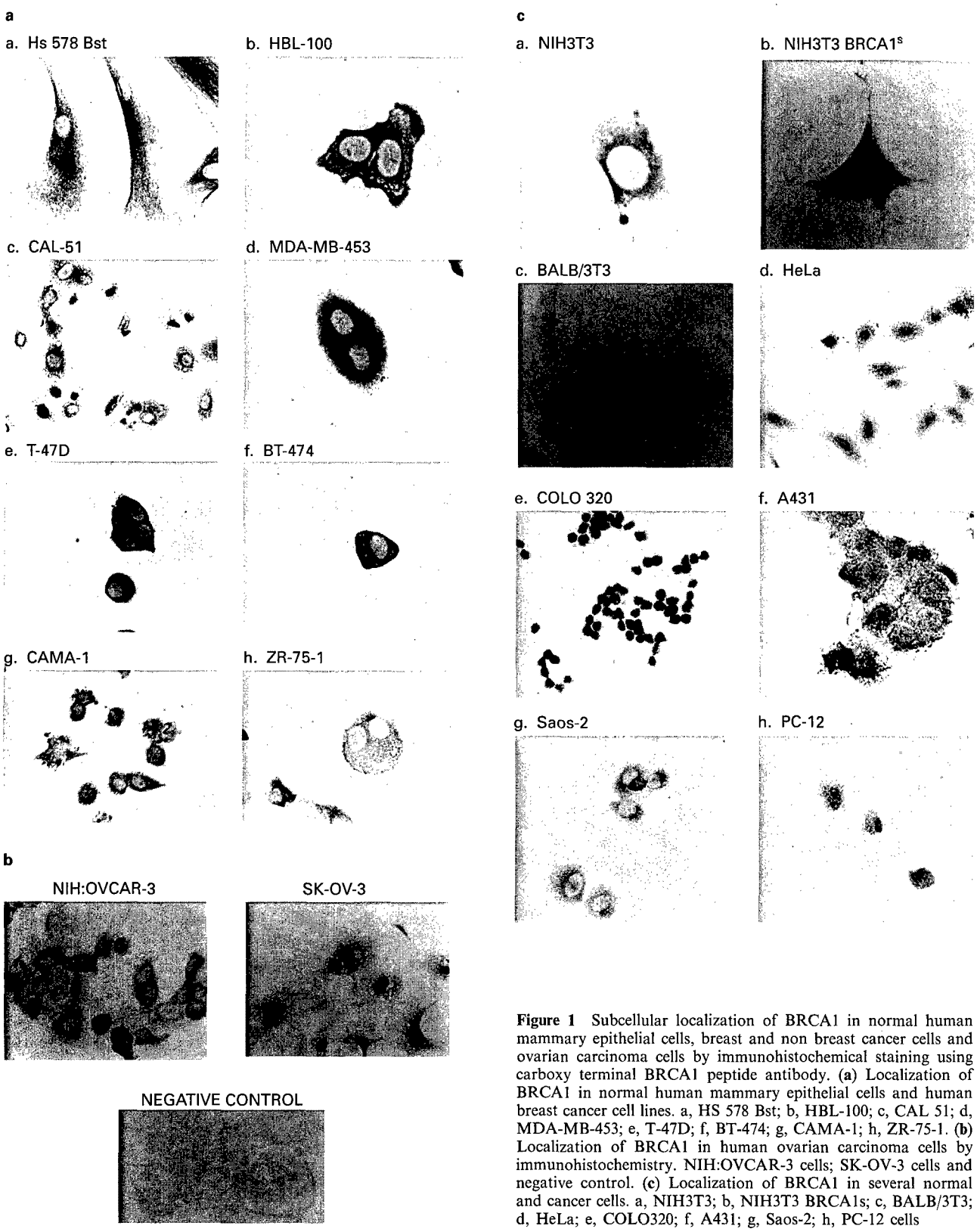


Figure 1 Subcellular localization of BRCA1 in normal human mammary epithelial cells, breast and non breast cancer cells and ovarian carcinoma cells by immunohistochemical staining using carboxy terminal BRCA1 peptide antibody. (**a**) Localization of BRCA1 in normal human mammary epithelial cells and human breast cancer cell lines. a, HS 578 Bst; b, HBL-100; c, CAL 51; d, MDA-MB-453; e, T-47D; f, BT-474; g, CAMA-1; h, ZR-75-1. (**b**) Localization of BRCA1 in human ovarian carcinoma cells by immunohistochemistry. NIH:OVCAR-3 cells; SK-OV-3 cells and negative control. (**c**) Localization of BRCA1 in several normal and cancer cells. a, NIH3T3; b, NIH3T3 BRCA1s; c, BALB/3T3; d, HeLa; e, COLO320; f, A431; g, Saos-2; h, PC-12 cells

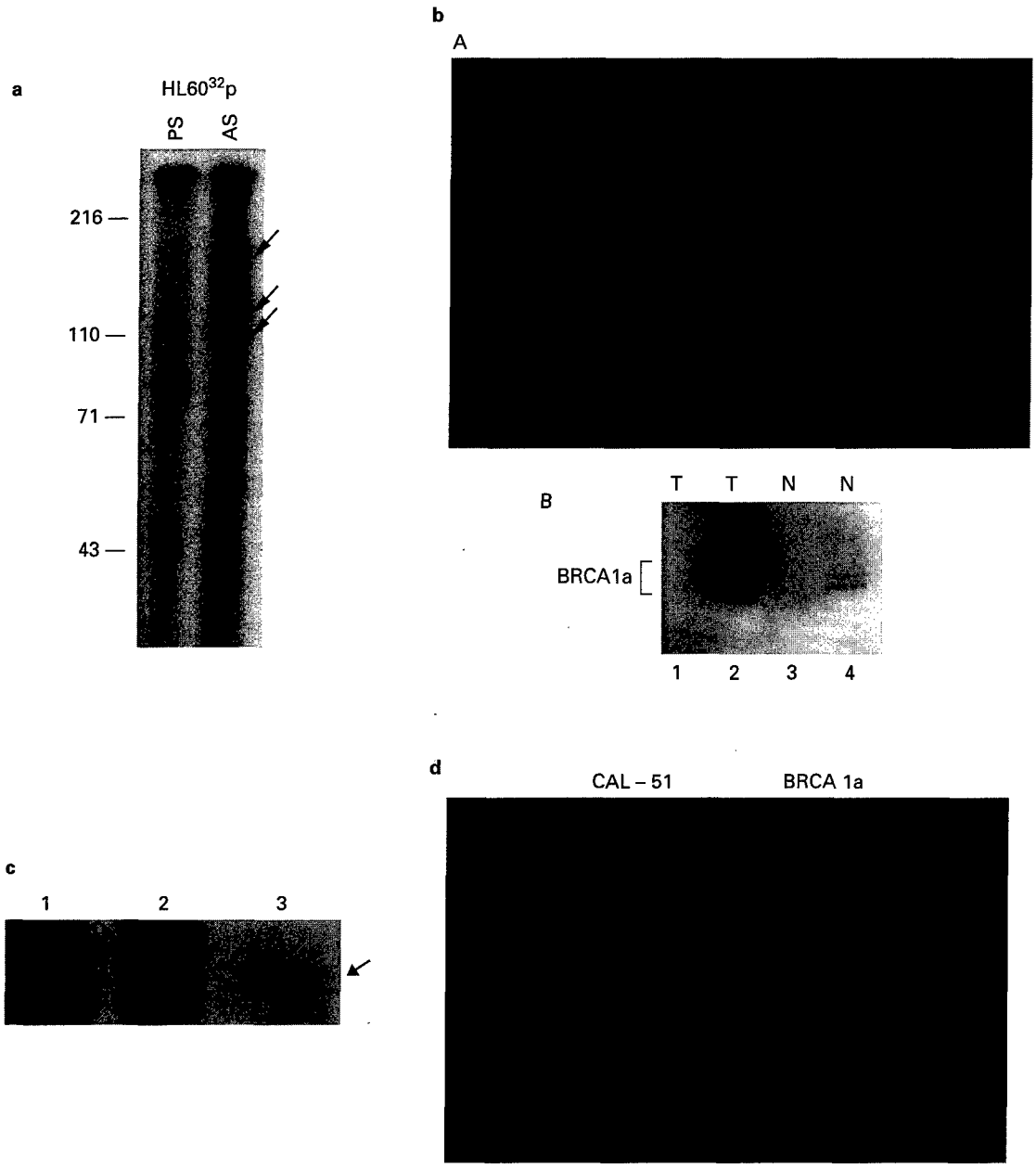


Figure 2 Subcellular localization and tyrosine phosphorylation of endogenous BRCA1 and FLAG-BRCA1a proteins in HL60, CAL-51 cells and COS cells by immunofluorescence and Western blot analysis. (**a**) Immunoprecipitation of ^{32}P-labeled HL60 cells with carboxy terminal BRCA1 peptide antibody. The three arrows represent the phosphorylated BRCA1 protein bands. (**b**) A, Immunostaining of COS cells transfected with FLAG-BRCA1a with FLAG antibody showing mostly cytoplasmic and weak nuclear staining; B, Western blot analysis of COS cells transfected with FLAG-BRCA1a with FLAG antibody shows BRCA1a to be localized both in the total (nucleus and cytoplasm) and nuclear fractions of COS cells. (**c**) Western blot analysis of FLAG-BRCA1a transfected COS cells shows BRCA1a to be a phosphoprotein containing phosphotyrosine. Lane 1 represents HL60 cells immunoprecipitated with pre-immune serum and Western blotted using phosphotyrosine antibody; lane 2 represents HL60 cells immunoprecipitated with carboxyterminal BRCA1 peptide antibody and then Western blotted using phosphotyrosine antibody; lane 3 represents COS cells transfected with CMV2 FLAG-BRCA1a, immunoprecipitated with FLAG antibody and Western blotted using phosphotyrosine antibody. The arrow represents the 110 kD BRCA1a protein. (**d**) Immunostaining of CAL-51 cells transfected with CMV2-FLAG-BRCA1a using FLAG antibody, shows BRCA1a to be localized mainly in the cytoplasm with weak nuclear staining

On prolonged incubation in serum free media for 48–72 h the staining was mostly nuclear with typical nuclear dot like pattern (data not shown). When the cells were refed with 10% serum for 24 h the BRCA1 protein was found to relocate back to the cytoplasm with weak nuclear staining similar to the situation seen in asynchronous serum fed NIH3T3 cells (Figure 3a). The same results were obtained using four different antibodies specific to different regions of BRCA1 protein, (data not given) thus ruling our the possibility that nuclear BRCA1 immunofluorescence might be an artifact due to starvation. Simultaneously, we have also determined the DNA content of these cells in the presence and absence of serum by FACS analysis to give a measure of the relative percentage of G1, S, G2/M cells in each sample. All these results seem to suggest that nuclear transport of BRCA1 is not cell cycle dependent and irrespective of the cell cycle state,

BRCA1 protein accumulates in the nucleus in the absence of serum and in the cytoplasm in the presence of serum, in NIH3T3 cells. We have also arrested the cells in G1 phase of the cell cycle by using drugs such as aphidicolin, mimosine and double thymidine block and studied the subcellular localization of BRCA1 protein. Our results suggest that the redistribution of the BRCA1 proteins to the nucleus in drug-treated cells is not as dramatic as seen with serum starved cells (Shao and Rao, unpublished results). It may be possible that accumulation of BRCA1 proteins in the nucleus of serum depleted NIH3T3 cells may be responsible for the induction of apoptosis seen previously in BRCA1a transfected NIH3T3 cells (Shao *et al.*, 1996). Two splice variants BRCA1 Δ672-4095 (Thakur *et al.*, 1997) and BRCA1 Δ11b which codes for a 110 kD protein (Wilson *et al.*, 1997) were both shown to be localized to the cytoplasm by immunostaining. Surprisingly, the authors found substantial levels of BRCA1 Δ11b to be in the nuclear fraction on immunoblotting (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b in COS cells. The molecular weight of the p110 kDa BRCA1a polypeptide reported in this and earlier studies (Shao *et al.*, 1996) is comparable to the 110 kDa BRCA1 Δ11b. Thus even though the reported nuclear localization signal (Thakur *et al.*, 1997; Wilson *et al.*, 1997) for BRCA1 is missing in BRCA1a and BRCA1b proteins, some of which still gets transported to the nucleus suggesting that both BRCA1a and BRCA1b may carry potential nuclear localization signals. It may be possible that there can be a serum-responsive regulatory protein that could be responsible for retention of BRCA1 protein within the cytoplasm of serum fed cells similar to NF-kB (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

Subcellular localization of BRCA1 proteins in breast and ovarian cancer cells grown in the presence and absence of serum

Since all these experiments were done in mouse fibroblasts, which may not be physiologically relevant to BRCA1 function we studied the subcellular distribution of BRCA1 in asynchronous and growth arrested serum deprived normal human breast epithelial cells Hs578 Bst, breast cancer cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cells NIHOVCAR-3, using different BRCA1 specific polyclonal antibodies by indirect immunofluorescence and immunohistochemical methods. We have used only those cell lines in which BRCA1 was found to be localized to the cytoplasm in asynchronous conditions. Our results suggest BRCA1 to be localized mainly in the cytoplasm of serum fed asynchronous normal breast epithelial cells Hs578 Bst, breast tumor cells HBL-100, ZR-75-1, CAMA-1 and ovarian carcinoma cell NIHOVCAR-3 (Figure 3b–f) and predominantly in the nucleus of growth arrested serum deprived Hs578 Bst, HBL-100, ZR-75-1 CAMA-1 and ovarian carcinoma cell line NIHOVCAR-3 (Figure 3b–f). All these results suggest that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is controlled by the extracellular environment with serum growth factors inhibiting the nuclear transport of the BRCA1 protein. It remains to be seen whether posttranslational modification (such as phosphorylation, etc.) of BRCA1 proteins plays a role in the subcellular localization of these proteins. The scenario we observe with BRCA1 is different to that seen for the c-Fos protein whose nuclear translocation appears to depend on the continuous stimulation of cells by serum factors (Roux *et al.*, 1990).

Detection of cellular proteins that interact with GST-BRCA1 fusion protein

Since the subcellular localization of BRCA1 is dependent on the presence of serum factors, and to investigate whether BRCA1 protein-protein interactions are of biological significance in the growth inhibitory, tumor suppressor and death inducing functions of BRCA1 protein, we studied the interaction of BRCA1 with cellular proteins. The amino terminal region of BRCA1 contains a zinc ring finger domain (Miki *et al.*, 1994) which are known to interact with DNA/RNA either through direct binding or indirectly by mediating protein-protein interactions. We have expressed a fusion protein that contains GST and the zinc finger domain of BRCA1 (residues 1–76) in bacteria using the Gex 2T expression vector system. In order to detect cellular proteins that interact with GST-BRCA1 fusion protein, whole cell lysates of human breast cancer cells ZR-75-1 or CA1-51 metabolically labeled with ^{35}S-methionine were incubated with either GST or the GST-BRCA1 fusion protein immobilized on glutathione-agarose beads (GSH-beads). The beads were washed, lysed in SDS sample buffer and subjected to SDS–PAGE. SDS–PAGE analysis of the bound complex revealed bands with relative molecular weight of $\approx$32 kD and $\approx$65 kD which bound exclusively to the GST-BRCA1 fusion protein and not to GST (Figure 4a). These bands were consistently detected in ZR-75-1, CAL-51 and HL 60 cell lysates and were designated as p65 BIP and p32 BIP respectively (data not shown).

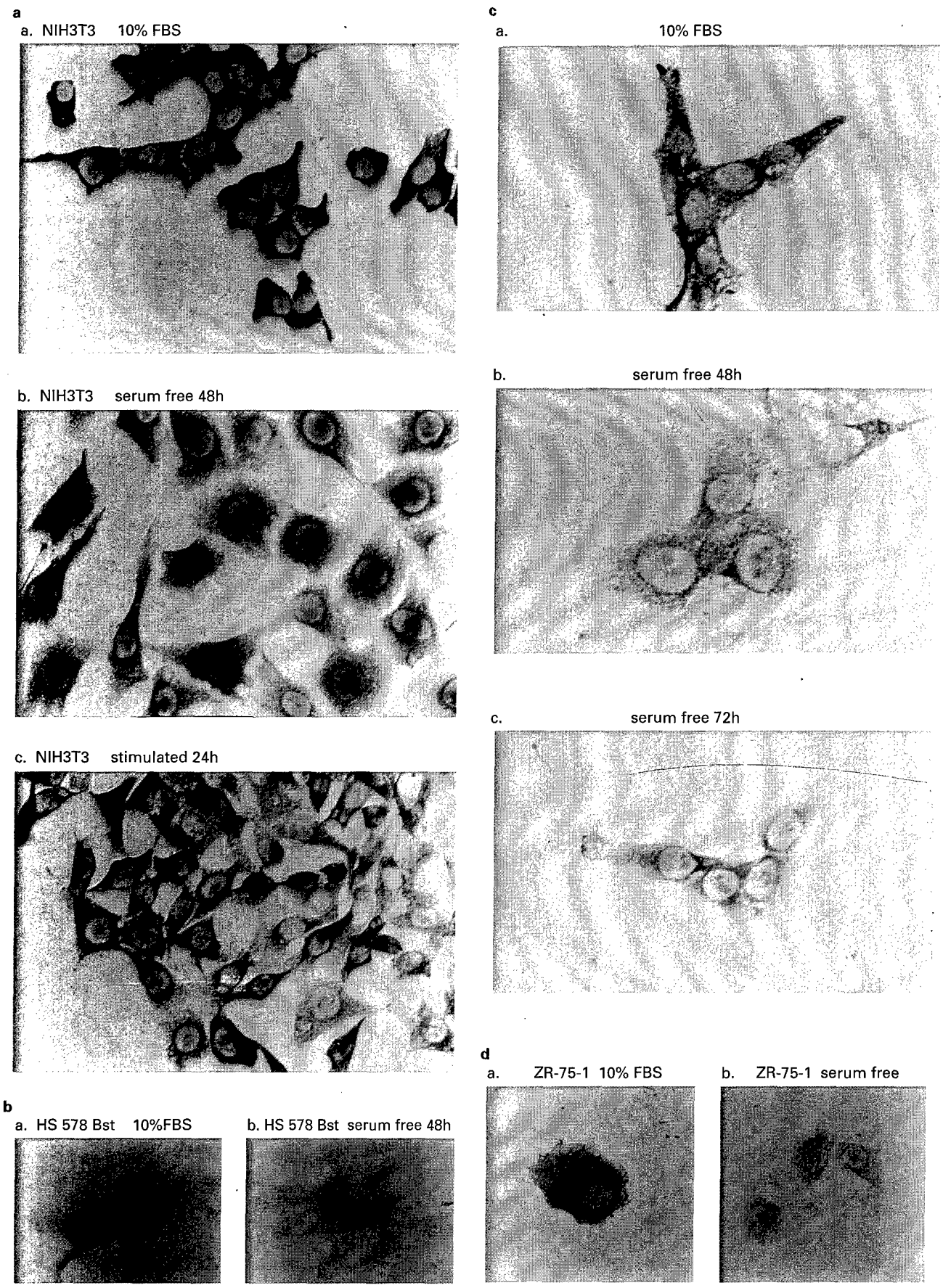
Direct association of p65 BIP and p32 BIP with BRCA1

To determine whether p65 BIP and p32 BIP directly interact with BRCA1, we performed a far Western blot analysis. The BIP complexes prepared from CAL-51 cell extract were separated on SDS–PAGE, transferred onto a nitrocellulose membrane, and probed with ^{32}P-labeled GST-TK-BRCA1 fusion protein. Since GST-TK-BRCA1 contains consensus phosphorylation site for protein kinase C at the amino terminal end, the purified protein can be ^{32}P-labeled by an *in vitro* protein kinase reaction. GST-BRCA1 bound to both p65 BIP and p32 BIP unlike GST protein (Figure 4b). These results suggest that both p65 BIP and p32 BIP bind strongly to BRCA1 even under stringent conditions without any mediating proteins (Figure 4b).

Expression of BRCA1 binding proteins in different cell lines

To determine the distribution of BRCA1-binding protein in various cell lines, we metabolically labeled

promyelocytic cell line HL60, breast cancer cell lines ZR-75-1 and CAL-51 cells and performed the GST-pull down assay as described previously. Both p65 BIP and p32 BIP were detected in all cell lysates examined (data not given) although at variable levels suggesting the ubiquitous expression of p65 BIP and p32 BIP.



Association of BRCA1 proteins with E2F, cyclins/CDKs

Recently, cdk-2 and other kinases associated with cyclins D and A were shown to phosphorylate BRCA1, suggesting a role for cdk's in regulating the activity of BRCA1 proteins (Chen, 1996a) and the growth inhibitory function of BRCA1 also suggested a role in cell cycle control (Holt *et al.*, Rao *et al.*,

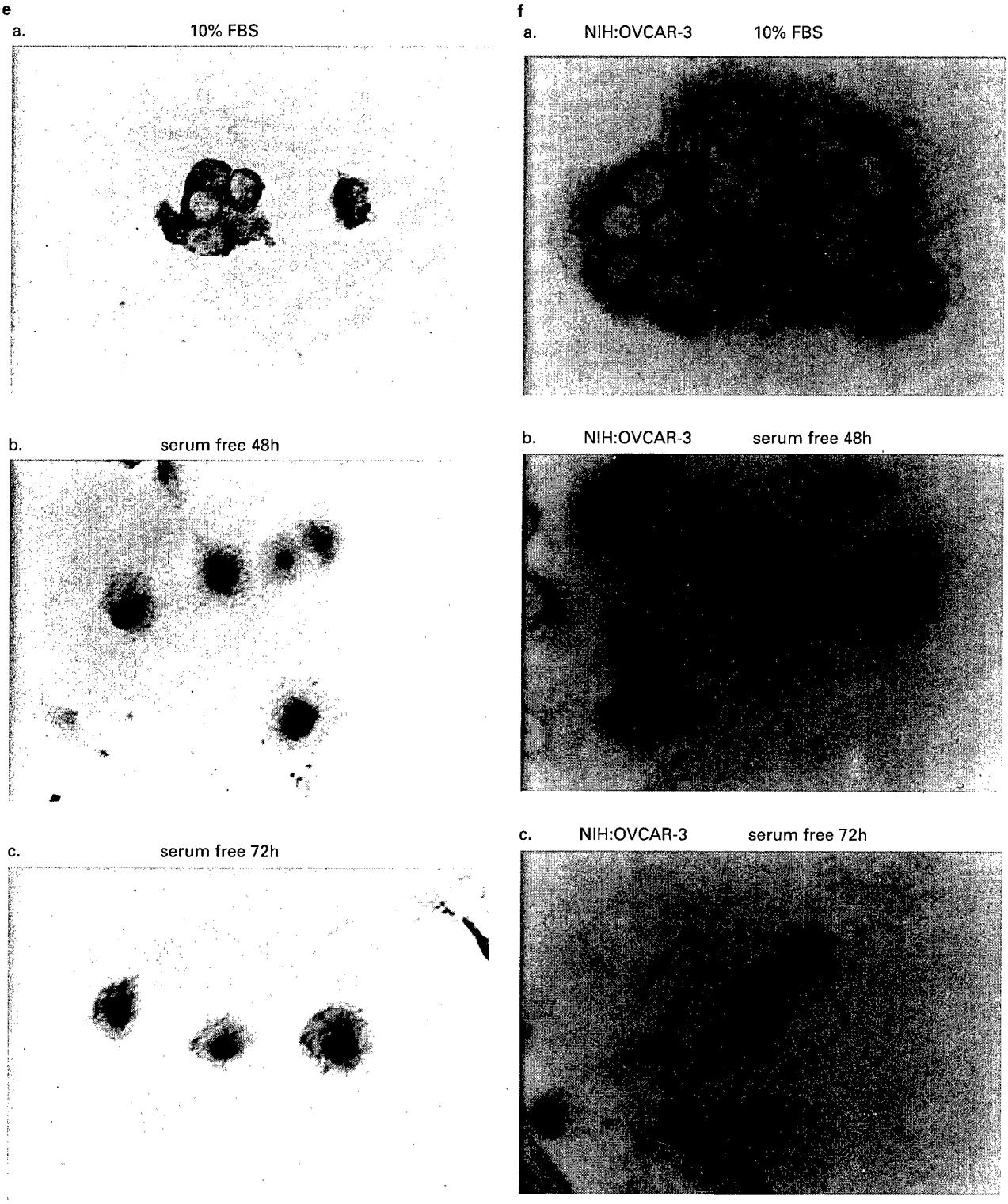
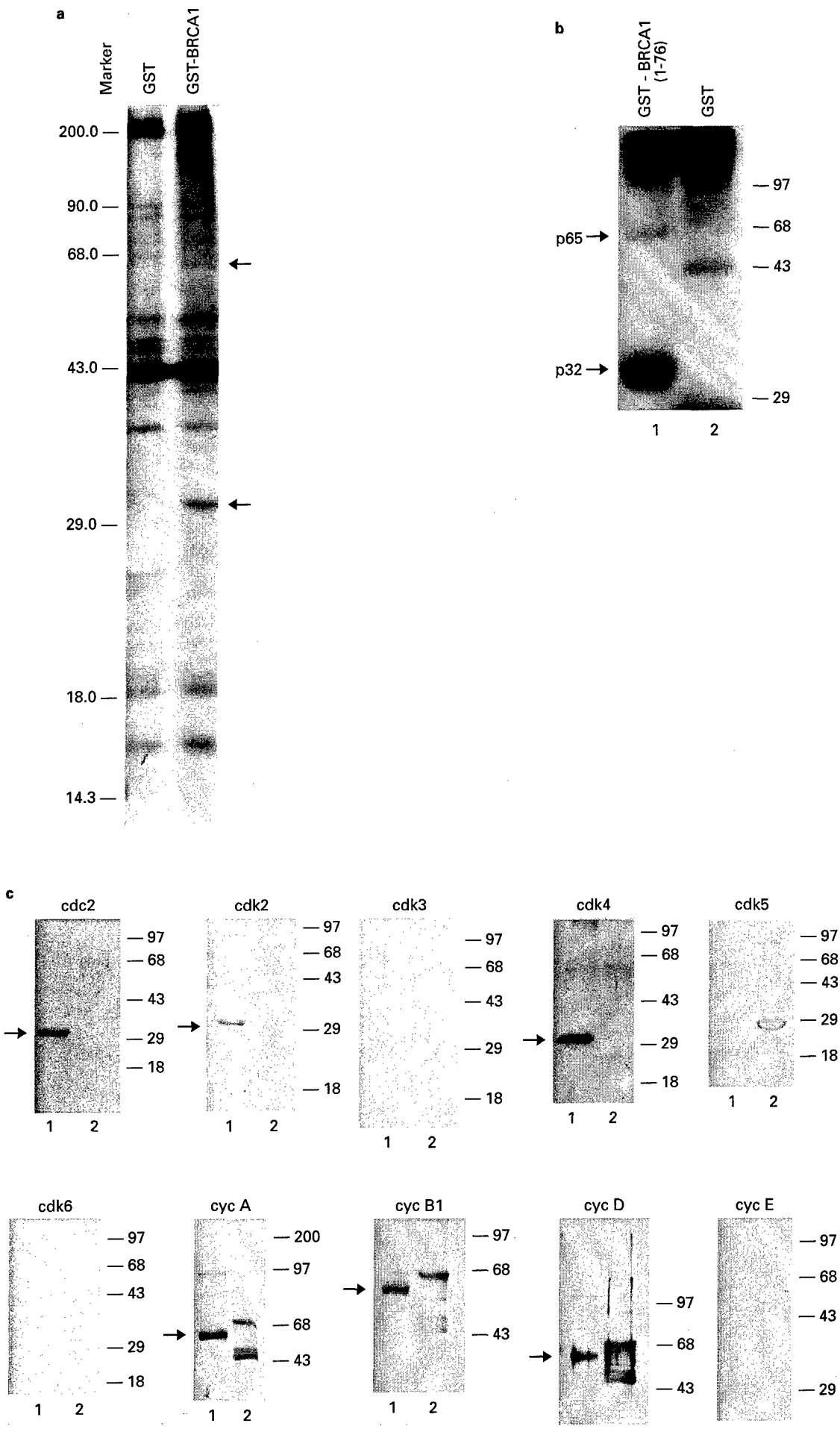


Figure 3 BRCA1 localizes to the nucleus in serum deprived NIH3T3, normal mammary epithelial cells, breast and ovarian cancer cells by immunohistochemical analysis. Intracellular localization of BRCA1 protein in serum-starved and serum fed NIH3T3 cells. (**a**) using amino-terminal BRCA1 peptide antibody. The above results were also confirmed using carboxy terminal BRCA1 peptide antibody, and an antibody raised against recombinant BRCA1 protein (aa 1–331), ethyl green was used as a positive control for nuclear staining (data not given). (**b**) BRCA1 redistributed to the nucleus in serum deprived normal human mammary epithelial cells HS578 Bst using immunohistochemical analysis. (**c**) BRCA1 translocated to the nucleus in serum deprived human breast cancer cells HBL-100. (**d**) ZR-75-1 cells, and (**e**) CAMA-1 cells using immunohistochemical analysis. (**f**) BRCA1 was found to translocate to the nucleus of serum deprived human ovarian carcinoma cell line NIHOVCAR-3. The above results were repeated using three different BRCA1 specific antibodies


a
Marker
GST
GST-BRCA1
200.0
90.0
68.0
43.0
29.0
18.0
14.3
b
GST - BRCA1 (1-76)
GST
p65
p32
97
68
43
29
1
2
c
cdc2
cdk2
cdk3
cdk4
cdk5
cdk6
cyc A
cyc B1
cyc D
cyc E
200

unpublished results). Since tumor suppressor genes like Rb regulate cell cycle by interaction with transcription factor E2F, cyclins/cdk's (Lees *et al.*, 1992; Kato *et al.*, 1993; Ewen *et al.*, 1993; Weinberg, 1995) and the sizes of BIPs are close to E2F, cyclins/CDKs, we speculated whether BRCA1 could similarly associate with E2F, cyclins/CDKs. We therefore incubated cell lysates obtained from CAL-51 cells with GST-immobilized on GSH-beads and BRCA1 fusion protein conjugated GSH-beads. The beads were then washed and heated in SDS sample buffer. The BIP complexes were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and probed with antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B1, cyclin D1, cyclin E, E2F-1, E2F-2, E2F-3, E2F-4 and E2F-5 as described previously (Rao *et al.*, 1996). The BIP complexes were recognized by antibodies specific to cdc2, cdk-2, cdk-4, cyclin D1, cyclin A, cyclin B1 (Figure 4c) and E2F-4 (Figure 4d), but not to cdk3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2, E2F-3 and E2F-5. All these results suggest association of BRCA1 with cyclin A, D1 and B1, cdc2, cdk-2, cdk-4 and E2F-4, but not to cdk-3, cdk-5, cdk-6, cyclin E, E2F-1, E2F-2 and E2F-5.

Tyrosine phosphorylation and kinase activity of BRCA1 interacting proteins

We next wanted to examine whether p32 BIP and p65 BIP contain phosphotyrosine. The BIP complexes obtained from CAL-51 cells were immunoblotted with a phosphotyrosine antibody (Figure 4d). Band corresponding to p32 was detected (Figure 4d) indicating that p32 BIP contains phosphotyrosine, a characteristic specific to cyclin associated protein kinases. We have also tested the BRCA1 immunoprecipitates from CAL-51 cells for kinase activity. Our

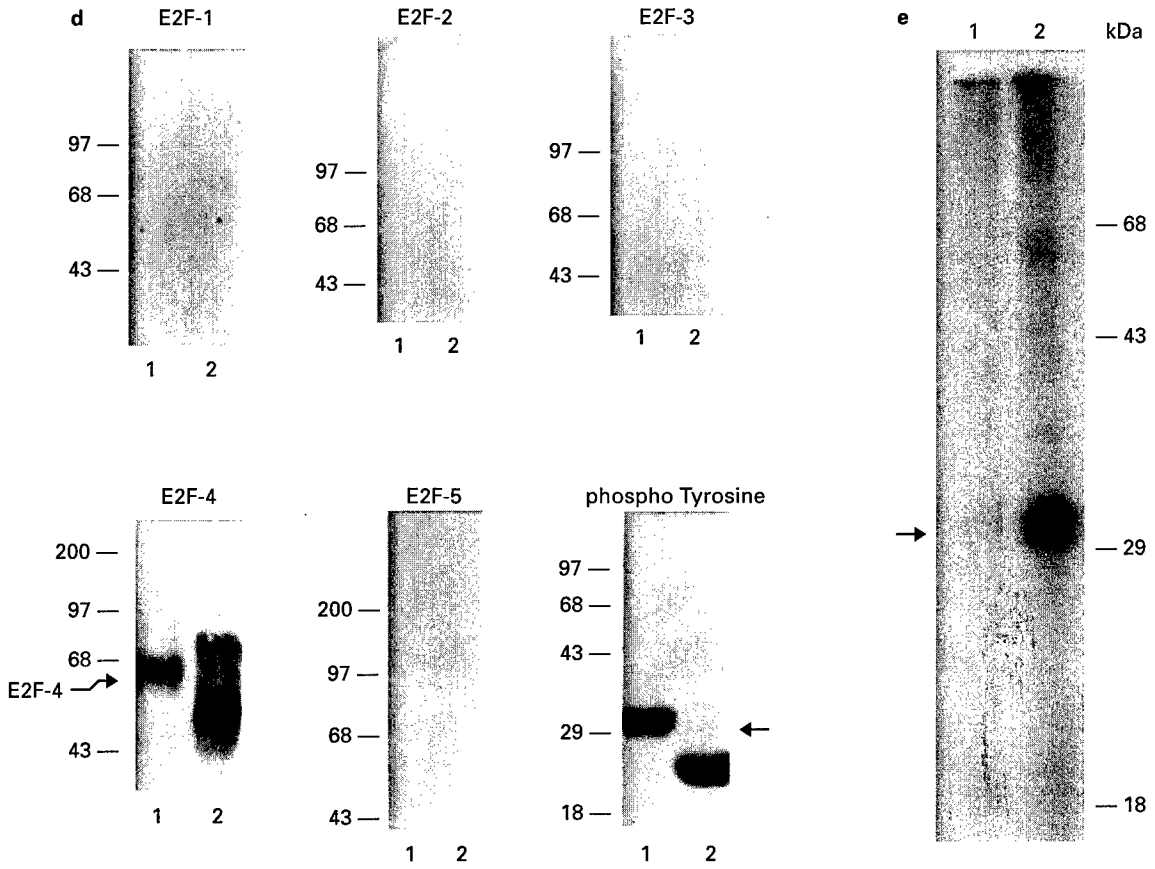
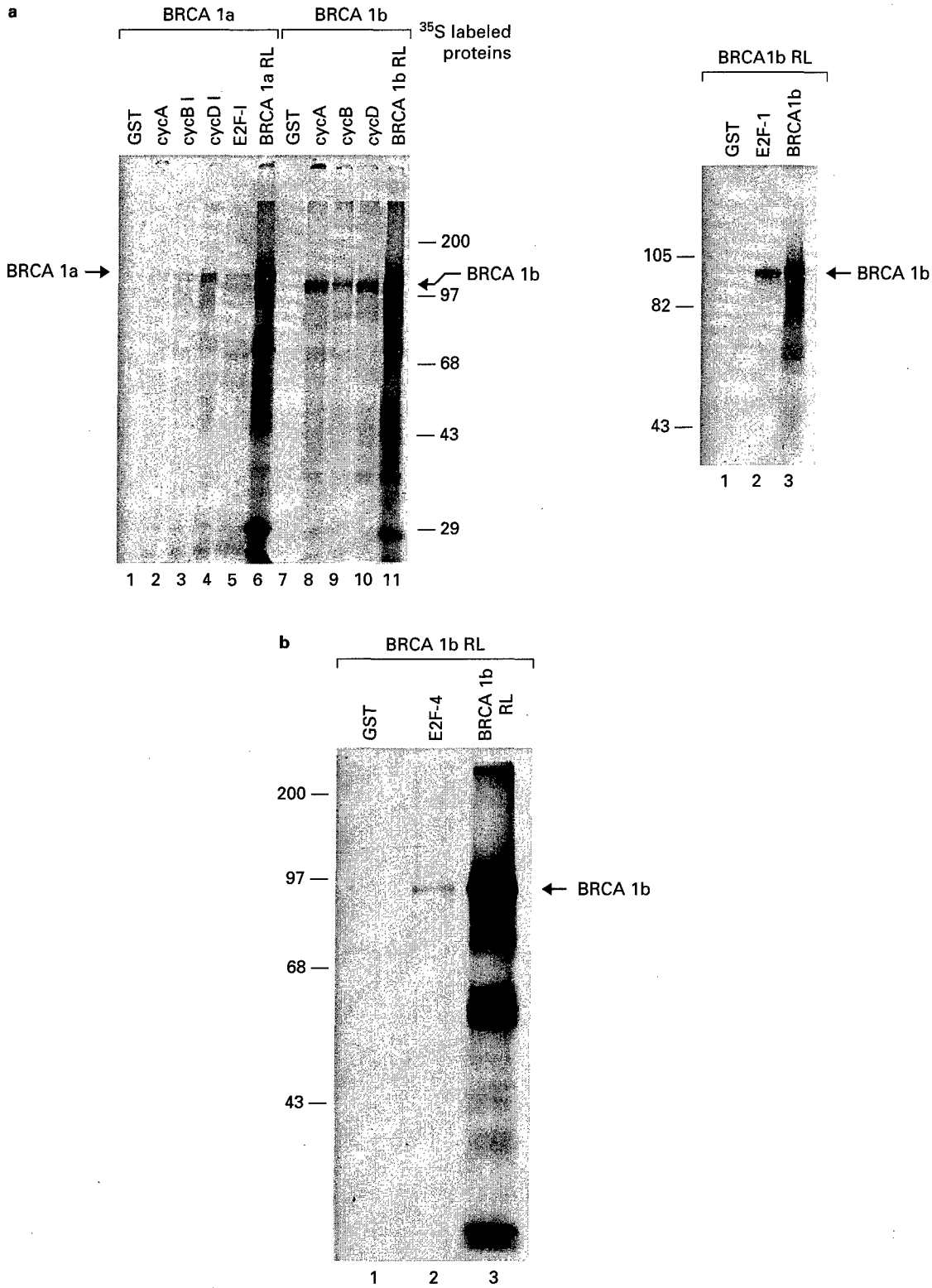


Figure 4 Identification of BRCA1 interacting proteins (BIP) that associate with E2F's cyclins/CDKs in breast cancer cells. (**a**) Detection of BRCA1-binding proteins. Lysates of ^{35}S-methionine-labeled CAL-51 cells were incubated with either GST or the GST-BRCA1 fusion protein immobilized on GSH beads. The bound proteins were washed, subjected to 10% SDS–PAGE and visualized by fluorography. The position of the major ≈p32 BIP complex is indicated on the right with a solid arrow and the minor ≈p65 BIP complex is represented by a small arrow on the right. The positions of the ^{14}C-labeled protein standards and their sizes are shown on the left. (**b**) Direct binding of BRCA1 with p32 BIP and p65 BIP by far Western blot analysis. CAL-51 cell extracts were passed through GST and GST-BRCA1 immobilized on glutathione beads. The protein complexes were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. After blocking, the filters were incubated with the ^{32}P-labeled GST-BRCA1 fusion protein. The arrows represent the p32 and p65 BIP proteins. (**c**) Association of BRCA1 with cyclin/cdk's. Unlabeled cell extract from CAL-51 cells were incubated with GST conjugated GSH-BRCA1 beads (lane 1) or with GST-conjugated GSH-beads (lane 2). The beads were then washed and subjected to 10% SDS–PAGE. The proteins were transferred onto nitrocellulose membrane and subjected to Western blot analysis using antibodies specific to cdc2, cdk2, cdk3, cdk4, cdk5, cdk6, cyclin A, cyclin B, cyclin D and cyclin E obtained from Santa Cruz Biotechnology. (**d**) Association of BRCA1 with transcriptional factor E2F-4 and the p32 BIP complex are tyrosine phosphoproteins. CAL-51 cell extracts were passed through GST-BRCA1 GSH beads (lane 1) or GST-GSH beads (lane 2) and the bound proteins were subjected to SDS–PAGE and Western blot analysis using antibodies specific to E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and phosphotyrosine obtained from Santa Cruz Biotechnology. The arrows represent E2F-4 and tyrosine phosphorylated p32 BIP proteins respectively. (**e**) Kinase activity of BRCA1 immunocomplexes on histone H1. Lane 1, buffer control; lane 2, histone H1. The arrow represents phosphorylated histone H1

results demonstrate that these immunocomplexes show histone H1 kinase activity confirming the association of BRCA1 with cyclins/cdk kinase complexes (Figure 4e).

In vitro *interactions of BRCA1a, BRCA1b with cyclins, E2F and CDKs*

To examine the binding of cyclin A, cyclin B1, cyclin D1, E2F-1 and E2F-4 with BRCA1a and BRCA1b *in vitro*, we inserted the cDNA sequences encoding BRCA1a and BRCA1b into pcDNA3 expression vectors (Rao *et al.*, unpublished results). *In vitro* transcription and translation of BRCA1a and BRCA1b in the presence of [^{35}S]methionine generated radiolabeled BRCA1a and BRCA1b polypeptides of ~110 and ~100 kD respectively (Figure 5a lane 6 and 11). These radiolabeled proteins were passed through GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-



E2F-1, GST-E2F-4 and GST respectively. Both *in vitro* translated BRCA1a and BRCA1b specifically bind to GST-cyclin A, GST-cyclin B1, GST-cyclin D1, GST-E2F-1 and GST E2F-4 unlike GST alone (Figure 5a, b). The BRCA1a splice variant bound at a reduced level compared to BRCA1b to all these different proteins. It may be possible that phosphorylation may regulate the binding of BRCA1a and BRCA1b proteins to cyclins, cdk's and E2F transcriptional factors. To confirm our results, further GST and GST E2F-1 fusion proteins were subjected to far Western blot analysis using ^{32}P-labeled amino-terminal BRCA1 (GST-BRCA1a amino acids 1–76, numbering from first ATG codon) fusion protein. The GST E2F-1 fusion protein band hybridized specifically to BRCA1 (Figure 5c). To further confirm the results, a fragment of BRCA1 encoding the amino-terminal 182 amino acids (amino acids 1–182) was *in vitro* translated and assayed for binding to GST-E2F-1 protein. The BRCA1 polypeptides bound specifically to GST-E2F-1 (Figure 5c). In a reciprocal assay, we *in vitro* translated the full length human E2F-1 cDNA and assayed it for binding to GST-BRCA1 zinc finger fusion protein (amino acids 1–76). The E2F-1 polypeptide bound very weakly to GST BRCA1 (Figure 5c). These results suggest that the amino-terminal 76 amino acids of BRCA1 were sufficient to provide specific association with E2F-1. The amount of E2F-1 and E2F-4 that got bound to BRCA1 appeared to be relatively low. It may be possible that E2Fs may need to associate with other proteins in order to bind efficiently to BRCA1. Similarly, we have *in vitro* translated human cdc2, cdk2, cdk4 and cdk5. The proteins thus generated were assayed separately for binding to GST and GST-BRCA1 (amino acids 1–76) fusion proteins. GST-BRCA1 specifically bound to cdc2 and cdk2 (Figure 5d) but not to cdk4 and cdk5 (data not given). All these results suggest that BRCA1 zinc finger domain can interact directly with cdc2 and cdk2.

Discussion

In the present report, we have presented data regarding the subcellular localization; phosphorylation and protein-protein interactions of BRCA1 and two BRCA1 isoforms, BRCA1a and BRCA1b. Initially, we have generated polyclonal antibodies against different regions of the human BRCA1 protein and used them to analyse BRCA1 expression in several normal, breast and ovarian cancer cells grown under different serum culture conditions. Our results suggest that BRCA1 proteins accumulate in the nucleus in the

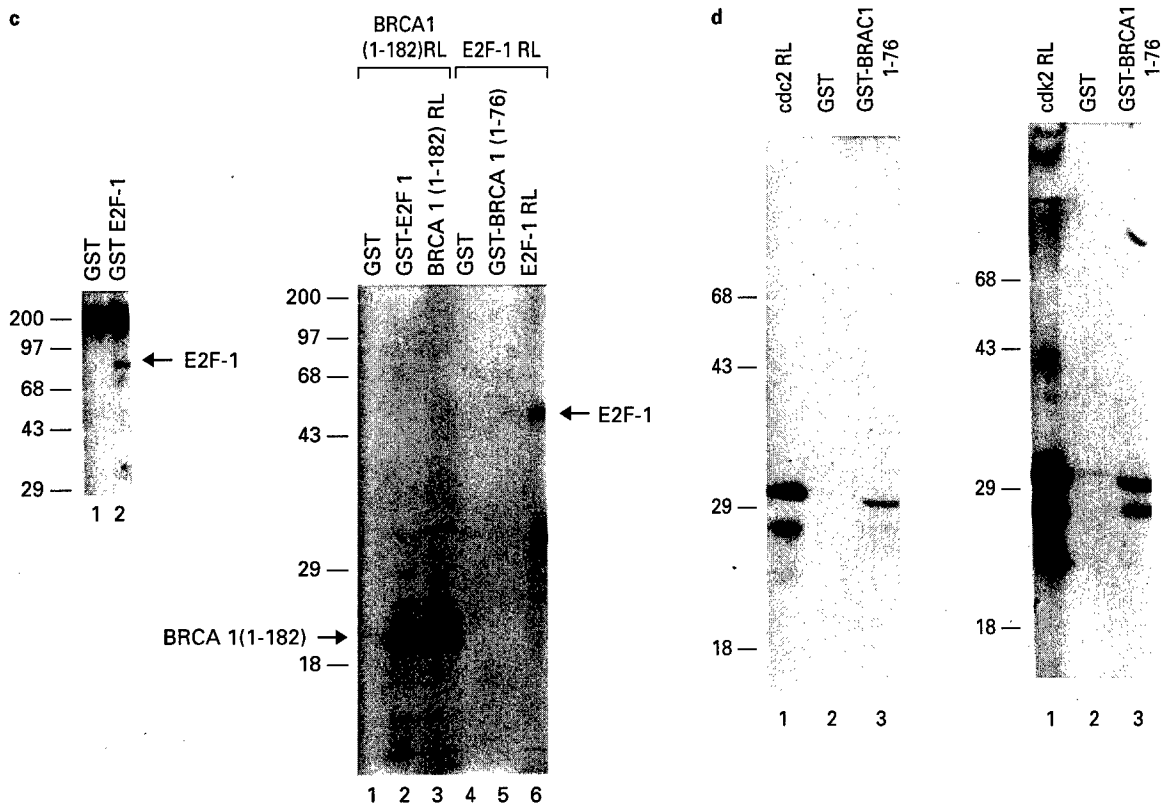


Figure 5 Direct physical interaction *in vitro* of BRCA1a and BRCA1b with cyclins, CDKs, E2F-1 and E2F-4 transcriptional factors. (**a**) *In vitro* translated BRCA1a and BRCA1b bind to GST-cyclin A, GST-Cyclin B1, GST-Cyclin D1, GST-E2F-1, 2 μl of the *in vitro* translated BRCA1a and BRCA1b were run as controls. Arrows represent *in vitro* translated BRCA1a (p110) and BRCA1b (p100). (**b**) *in vitro* translated BRCA1b binds to GST-E2F-4. Arrow represents *in vitro* translated BRCA1b (p100). (**c**) The panel on the left represents far Western blot analysis of BRCA1 (amino acids 1–76) binding directly to E2F-1. The arrow represents the position of GST-E2F-1 fusion protein. The panel on the right represents *in vitro* translated BRCA1 (amino acids 1–182) and *in vitro* translated full length E2F-1 binding to GST-E2F1 and GST-BRCA1 (amino acids 1–76) respectively. The arrows represent *in vitro* translated BRCA1 (amino acids 1–182) and E2F-1. (**d**) *In vitro* translated full length cdc2 and cdk2 bind to GST-BRCA1 (amino acids 1–76). 2 μl of *in vitro* translated cdc2 (left panel) and cdk2 (right panel) were loaded as controls

absence of serum and in the cytoplasm in the presence of serum in NIH3T3 cells and some breast and ovarian cancer cells. It may be possible that the nuclear or cytoplasmic transport of BRCA1 is not spontaneous but is controlled by the extracellular environment with serum inhibiting the nuclear transport of the BRCA1 protein. Alternatively, it may be possible that a protein factor present in the cytoplasm of serum fed cells could be responsible for retention of BRCA1 protein within the cytoplasm, similar to NF-KB (Baeuerle and Baltimore, 1988) and the levels of which could determine the subcellular localization of BRCA1 protein. We are presently investigating these possibilities.

The BRCA1 cDNA codes for a 1863 amino acid protein (Miki *et al.*, 1994) with an amino terminal zinc ring finger motif and two putative nuclear localization signals, suggesting that it might be a nuclear protein (Miki *et al.*, 1994; Lane *et al.*, 1995). There have been several discrepancies in the literature regarding the size and subcellular localization of BRCA1 (Chen *et al.*, 1995, 1996; Rao *et al.*, 1996; Wilson *et al.*, 1997; Scully *et al.*, 1996; Jensen *et al.*, 1996). Previously, we have reported BRCA1 proteins to be localized mainly in the nucleus and some in the cytoplasm of Rat-1 and MCF7 cells (Rao *et al.*, 1996). Subsequently, we have isolated two new alternately spliced BRCA1 transcripts referred to as BRCA1a (p110) and BRCA1b (p100) (Rao *et al.*, unpublished results) and developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1a proteins (Shao *et al.*, 1996). Our results indicate that over expression of BRCA1a induces apoptosis in NIH3T3 and MCF-7 cells after calcium ionophore treatment indicating that BRCA1 genes may play a critical role in the regulation of apoptosis (Shao *et al.*, 1996). In this report, we have studied the subcellular localization of BRCA1a and BRCA1b proteins using FLAG epitope tagged CMV promoter vectors. Our data using immunofluorescence and immunoblotting analysis indicate cytoplasmic and nuclear localization of FLAG BRCA1a and FLAG BRCA1b proteins. Recently, two other groups (Thakur *et al.*, 1997; Wilson *et al.*, 1997) have identified two BRCA1 splice variants BRCA1Δ672-4092 (which lacks exon 11) and BRCA1Δ11b (which lacks majority of exon 11) both of which were found to localize to the cytoplasm by immunostaining. These investigators have mapped a nuclear localization signal in exon 11 of BRCA1 which is missing in BRCA1Δ672-4095 and BRCA1Δ11b suggesting that splicing may regulate the function of BRCA1 by altering the subcellular localization of these proteins (Thakur *et al.*, 1997; Wilson *et al.*, 1997). Interestingly, BRCA1Δ11b was also found to be present in significant quantities in the nuclear fractions on immunoblotting analysis (Wilson *et al.*, 1997), similar to what we have observed with BRCA1a and BRCA1b proteins (this study). We have also found the BRCA1 proteins to accumulate in the cytoplasm in the presence of serum and in the nucleus in the absence of serum. All these results suggest that the nuclear localization of BRCA1 may be regulated by external stimuli, phosphorylation or protein-protein interactions. BRCA1 expression was shown to be high in tissues that are undergoing rapid growth and differentiation (Lane *et al.*, 1995; Marquis *et al.*, 1995). Recently the BRCA1 protein was also shown to be expressed and phosphorylated in a cell cycle dependent manner (Chen *et al.*, 1996). The BRCA1 m-RNA levels were found to be high in exponentially growing cells and in cells just prior to entry into S-phase but decreased upon growth factor withdrawal or after treatment with transforming growth factor β-1 (Gudas *et al.*, 1996) suggesting cell cycle regulation of BRCA1 expression. In an attempt to isolate proteins that interact with BRCA1, we have detected two cellular proteins (p65 BIP, p32 BIP) that specifically interact with BRCA1. Western blot analysis of BIP indicated association with E2F, cyclins and CDKs and *in vitro* translated BRCA1a and BRCA1b proteins interacted directly with transcription factor E2F, cyclins and CDK's suggesting a role for these proteins in regulating the biological activity of BRCA1 proteins.

In summary our results indicate BRCA1 gene products to be nuclear tyrosine phosphoproteins (Rao *et al.*, 1996; Chen *et al.*, 1995; this study) that translocate to the nucleus in the absence of serum, function as tumor/growth suppressors (Rao *et al.*, 1996; Rao *et al.*, unpublished results); Holt *et al.*, 1996), inducers of apoptosis (Shao *et al.*, 1996) and associate *in vitro* with E2F transcriptional factors, cyclins and cdk complex, suggesting a role for CDKs in regulating the biological activity of BRCA1. Several critical growth regulators like the product of the pRB, a tumor suppressor protein have been shown to associate with E2F both *in vivo* and *in vitro* (Weinberg, 1995) resulting in net inhibition of E2F-mediated transactivation and E2F reslease from pRB is an important event in the activation of genes required for S-phase entry (Weinberg, 1995). It may be possible that tumor suppressor proteins like BRCA1 may similarly interact with E2Fs and regulate cell proliferation. The interaction of BRCA1 zinc ring finger domain to E2Fs, cyclins/cdk complexes assigns a function for this domain in mediating protein-protein interaction. The function of BRCA1 zinc ring domain becomes important since it is the lcoation of some of the most frequently occurring mutations linked to breast and ovarian cancers. One of the BRCA1 mutations contains a frame shift in exon 2 (188 del 111; Miki *et al.*, 1994) which removes the zinc finger domain from the protein. The 185 del AG mutation, which is the most common BRCA1 mutation seen to date that occurs 1 in 100 Ashkenazi Jewish individuals (Shattuck-Eiden *et al.*, 1995) disrupts the BRCA1 gene product at the first residue of the C_3HC_4 domain. These frequent missense mutations Cys 61, Gly, Cys 64, Gly, and Cys 64 Tyr also disrupt the zinc finger domain structure. It may be possible that lack or impaired binding of the disrupted BRCA1 protein to E2F, cyclins/CDKs in patients with mutations in the zinc ring domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer. Future efforts will be directed towards showing the identity of p32 BIP and p65 BIP and identifying the cyclins/CDKs and E2Fs family members which specifically interact with BRCA1 *in vivo*. It remains to be seen whether the interactions we observe *in vitro* can be seen *in vivo* and if they are physiologically relevant.

Materials and methods

Cell lines

NIH3T3 cells and their derivatives, MCF-7, MDA-MB-453 and A431 cells were grown at 37°C in DMEM supplemented with 10% fetal bovein serum (FBS), 1% penicillin-streptomycin (PS); HS578 Bst cells were grown in DMEM supplemented with 10% FBS, 1% PS and 30 ng/ml EGF, CAL-51 cells were grown in DMEM containing 10% FBS, 1% PS, 0.6 μg/ml bovine insulin, 5×10^{-3} μg/ml transferrin and 146 mg/Liter glutamine; ZR 75-1 and COLO 320 were grown in RPMI 1640 supplemented with 10% FBS, 1% PS and 10 μg/ml bovine insulin; NIH:OVCAR-3 cells were cultured in RPMI 1640 supplememted with 20% FBS, 1% PS and 10 μg/ml bovine insulin; PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum and 5% FBS, 1% PS; SK-OV-3 and HBL 100 cells were grown in McCoy's 5a medium supplemented with 10% FBS and 1% PS; Saos-2 cells were grown in McCoy's 5a medium supplemented with 15% FBS and 1% PS; CAMA-1 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS and 1% PS. All the cell lines except CAL-51 cells were obtained from American type culture collection (Rockville, MD).

Immunochemistry

The different cell lines in the logarithmic stage of growth cultured in chamber slides were made quiescent in the presence of DMEM only for a period of 24–72 h. Cells were then washed in PBS and subjected to immunohistochemistry analysis using rabbit anti BRCA1 carboxyterminal or an amino terminal peptide antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal antibody generated against GST fusion proteins containing amino acids 1 to 76 and amino acids 1 to 331 of the BRCA1 protein as described previously (Rao *et al.*, 1996).

Plasmid construction and transfections

BRCA1a cDNA and BRCA1b cDNA (Rao, unpublished results) were subcloned into pFLAG-CMV vector (Eastman Kodak Company) by PCR. Purified DNA 5 μg for chamber slides and 20 μg for 100 mm petri dishes of pFLAG-CMV-2 expression vector or pFLAG-CMV-2 expression vector containing the BRCA1a cDNA were transfected into COS and CAL-51 cells using the Invitrogen kit according to the manufacturer's recommendations. After 48–72 h post transfection cells were processed for immunofluorescence analysis or the cell extracts were subjected to Western blot analysis using FLAG M2 antibody (Eastman Kodak Company) as described previously (Rao *et al.*, 1996).

Immunofluorescence

Immunofluorescence analysis was done as described previously (Rao 1996; Shao *et al.*, 1996) except with slight modifications. In brief, pFLAG-CMV-2 BRCA1a transfected COS and CAL-51 cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, followed by washing in PBS and PBS with 0.05% Triton X-100 and blocking in blocking solution (4% normal goat serum, 0.05% Triton X-100 in PBS) for 10 min at room temperature. The cells were incubated with primary anti FLAG M2 antibody diluted 1:100 for COS and CAL-51 cells, then washed three times with PBS, blocked with blocking solution for an additional 10 min at room temperature followed by incubation with the secondary antibody (FITC conjugated goat anti mouse IgG). After rinsing in PBS, the slides were mounted with mounting media and photographed on a confocal microscope.

Preparation of total nuclear extract and Western blot analysis

COS cells were harvested 48 h post transfection by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in RIPA buffer and the lysate was centrifuged at 14 000 r.p.m. for 30 min at 4°C. The supernatant was assayed for protein concentration by Bradford's method (Bio-Rad) and $\approx$50–100 μg of protein was subjected to Western blot analysis as described previously (Rao *et al.*, 1996). The nuclear extract was prepared as described (Hurst *et al.*, 1990). In brief, cells were lysed in nuclear extract buffer 1 (Hurst *et al.*, 1990), centrifuged at high speed for 1 min at room temperature. The crude nucleus was suspended in nuclear extract buffer II (Hurst *et al.*, 1990). Nuclear debris was removed by centrifugation for 1 min at room temperature. The supernatant was diluted by the addition of 20 mM HEPES (pH 7.4). The protein concentrations were determined by Bradford's method (Bio-Rad) and $\approx$50–100 μg of protein was subjected to Western blot analysis.

For Western blotting analysis $\approx$50–100 μg of cell/nuclear extract in SDS sample buffer were loaded on a 10% SDS–PAGE in Bio-Rad mini-protean II cell as described previously (Rao *et al.*, 1996). After electro transfer onto PVDF membrane, the FLAG-BRCA1a fusion protein was detected with anti-FLAG M2 antibody diluted 1:100 using Western exposure chemiluminescent detection system from Clonetech or ECL as described previously (Rao *et al.*, 1996).

Metabolic labeling of cells

Briefly, confluent 100 mm plates of HL 60 cells were labeled with ^{32}P-orthophosphoric for 4 h. The cells were lysed in radioimmunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti BRCA1 peptide or recombinant protein antibody or preimmune serum as described previously (Rao *et al.*, 1996). The samples were subjected to 10% SDS polyacryladmide gel electrophoresis and autoradiography. In some cases the cold HL60 cell lysates were subjected to immunoprecipitation using carboxyterminal BRCA1 peptide antibody and then subjected to Western blot analysis using phosphotyrosine antibody (Santa Cruz Biotechnology).

Expression and purification of GST fusion protein

Expression and purification of GST fusion proteins were described previously (Rao and Reddy, 1993) except with slight modification (Frangioni and Neel, 1993). Briefly, log phase cultures of *E. coli* BL21 (DE3) LysS transformed with the pGEX 2TK-BRCA1 (aa 1–76), pGEX2T-BRCA1 (aa 1–76), pGEX 2T-E2F-1, pGex2T-CycA, pGEX2T-cycB1, pGEX2T-cycD1, pGEX2T-E2F-4 plasmids were incubated with IPGT for 3 h. The cells were pelleted in STE buffer (Frangioni and Neel, 1993) containing 100 μg/ml lysozyme, 5 mM DTT, 1 mM PMSF and 2% Sarkosyl, sonicated on ice and centrifuged at 10 000 *g* for 10 min. To the supernatant Triton X-100 was added and applied to a glutathione sepharose 4B column (Pharmacia) and the GST-BRCA1 or GST-cyclins or GST-E2F fusion proteins were either left immobilized or eluted with elution buffer containing glutathione (Rao and Reddy, 1993). The GST-BRCA1 fusion proteins were labeled with ^{32}P as described (Kaelin *et al.*, 1992). In brief, the GST-TK-fusion protein probes were labeled in a 100 μl final volume containing 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 10 μCi of (γ-^{32}P) ATP, 1 μg GST-fusion protein and 100 units of cAMP dependent protein kinase (Sigma) on ice for 30 min.

GST pull down assay

CAL-51, ZR 75-1 or HL 60 cells were labeled with ^{35}S-methionine as described previously (Rao *et al.*, 1996). The cells were washed in cold phosphate buffered saline (PBS) and scrapped into 1 ml of TNN buffer (Takashima *et al.*, 1994) and lysed by rotating for 30 min at 4°C. The lysates were centrifuged at 14 000 *g* for 30 min and subjected to protein binding assay as described (Takashima *et al.*, 1994). For protein binding assay cell extracts were precleared overnight with GSH-beads and then incubated with either GST protein-conjugated GSH-beads or GST-BRCA1 (containing residue 1 to 76 of the BRCA1a protein). Proteins were incubated with GSH beads for 2 h at 4°C. The beads were then washed in TNN buffer and boiled in SDS sample buffer and loaded on a 10% SDS–PAGE. The gels were fixed, treated with enhance, dried and exposed to X-ray films. For *in vitro* binding experiments 10–20 μl of full length *in vitro* translated BRCA1a, BRCA1b, BRCA1 (amino acids 1–182), cdc2, cdk2, cdk4, cdk5 and E2F-1 were tested for binding to GST-E2F-1 or GST-E2F-4 as described previously for GST pull down assay.

Immunoprecipitation and in vitro *kinase assays*

CAL-51 cells were lysed in 1 ml TNN buffer and immunoprecipitated with recombinant BRCA1 polyclonal antibody (amino acid 1–76) as described previously (Rao *et al.*, 1996). The immunoprecipitates were washed in kinase buffer and measured for kinase activity toward histone H1 as described previously (Makela *et al.*, 1994).

Far Western blot analysis

Far Western blot analysis was done as described previously (Kaelin *et al.*, 1992; Singh *et al.*, 1989) except with slight modifications. In brief, after transfer the nitrocellulose membrane was washed in 1 × HBB buffer (Singh *et al.*, 1989) and treated sequentially with 1 × HBB buffer containing different concentrations of guanidine HCl ranging in concentration from 6 M to 0.19 M. The membrane was hybridized in Hyb 75 buffer (Kaelin *et al.*, 1992) containing 0.1 mM $ZnCl_2$ and ^{32}P-labeled GST-TK-BRCA1 protein (10^6 c.p.m./ml) overnight at 4°C. Subsequently the membrane was washed in Hyb 75 buffer, air dried and exposed to X-ray film.

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Differential transcriptional activation by the N-terminal region of BRCA1 splice variants BRCA1a and BRCA1b

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Abstract. The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor in human breast cancer cells. BRCA1 protein contains an amino-terminal zinc finger motif and a carboxy-terminal acidic region. Recently, the carboxy-terminal region of BRCA1 and the amino-terminal region of BRCA2 proteins were shown to function as transactivation domains when fused to GAL4 DNA binding domain. We have recently isolated and characterized two new naturally occurring variants of BRCA1 (BRCA1a/p110 and BRCA1b/p100) which are phosphoproteins containing phosphotyrosine that associate with E2F transcriptional factors, cyclins and cyclin dependent kinases indicating a role for BRCA1 proteins in cell-cycle regulation. Here we show for the first time that the amino-terminal region of BRCA1a (BNT) but not BRCA1b can also function as a transcriptional activator when fused to GAL4 DNA binding domain. Thus, BRCA1/1a proteins contain two autonomous transcriptional activation domains, one at the amino-terminal region (BNT) and the other at the carboxy-terminal region (BCT). BRCA1b retains only the BCT domain since it has lost part of the potential BNT domain as a result of alternative splicing. Our results also suggest the presence of an inhibitory domain at the carboxy terminal region of BRCA1 and BRCA1a proteins (BID). Thus, BRCA1b protein may function as a dominant negative variant that could regulate the transcriptional activity of BRCA1/BRCA1a proteins and hence may serve as a marker for identifying individuals with greater potential for developing breast cancer. It may be possible that loss of transcriptional activation or protein-protein interactions in patients with mutations in the amino terminal zinc finger domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer.

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Key words: BRCA1a; BRCA1b; transcriptional activation, zinc finger, breast cancer

Introduction

Breast cancer is one of the most common malignancies affecting women in the United States. The breast and ovarian cancer susceptibility gene BRCA1 was mapped by genetic linkage analysis to human chromosome 17q21.3 (1-5) establishing the presence of a breast cancer susceptibility gene. Mutations in the BRCA1 gene account for 45% of the inherited breast and ovarian cancers and are found in 90% of families with high incidence of both breast and ovarian cancers. The BRCA1 cDNA codes for a 1863 amino acid protein with an amino-terminal zinc finger domain and a carboxy-terminal acidic region typical of several transcriptional factors (3). The BRCA2 gene which also encodes a large protein was found to be mutated in 45% of familial breast cancers (6). Previously, the carboxy-terminal region of BRCA1 (7,8, Rao *et al*, unpublished data) and the amino-terminal region of BRCA2 proteins (9) were shown to activate transcription when fused to the GAL4 DNA binding domain suggesting a function for BRCA1 in transcriptional activation. Several groups have characterized the developmental patterns of expression of murine BRCA1 (10-13). BRCA1 expression was found to be high in rapidly proliferating tissues (10,11) particularly those undergoing differentiation suggesting a role for BRCA1 in cellular growth and differentiation. BRCA1 was also shown to be essential for early embryonic proliferation and development (14-16). The BRCA1 protein is a nuclear phosphoprotein (17-21) which localizes to discrete nuclear dots during S phase (19,22,23). These structures contain two additional proteins Rad51 and BARD1, both of which associate with BRCA1 *in vivo* (23,24) suggesting a role for BRCA1 in DNA repair and the maintenance of genome stability. Recently, DNA damaging agents were shown to alter the subcellular localization and phosphorylation state of p220 BRCA1 protein suggesting a role in S phase DNA damage-dependent cell cycle checkpoint response (25). Our results suggest that BRCA1 proteins accumulate in the nucleus in the absence of serum and in the cytoplasm in the presence of serum in some cells revealing a new type of regulation that may modulate the activity of the BRCA1 gene (20). Several other functions have been associated with BRCA1 for instance BRCA1 when overexpressed in breast and ovarian cancer cells results in growth inhibition both *in vitro* and *in vivo* in breast cancer cells (26, Rao *et al*, unpublished data). Conversely, inhibition of BRCA1 expression by antisense RNA in mouse fibroblasts

or by antisense oligonucleotides in breast cancer cells resulted in transformation of mouse fibroblasts as well as increasing the rate of growth of breast cancer cells (18,27) implicating a direct role for BRCA1 in growth and tumor suppression. We have recently found the BRCA1 splice variant BRCA1a to induce apoptosis in human breast cancer cells (28) suggesting a role for BRCA1 proteins in the regulation of apoptosis. All these results seem to indicate BRCA1 to be a truly multi-functional protein.

Previously, we have isolated and characterized two new BRCA1 splice variants, BRCA1a (p110) and BRCA1b (p100) which are phosphoproteins containing phosphotyrosine (20). BRCA1a and BRCA1b proteins are localized both in the cytoplasm and nucleus and associate via their amino-terminal zinc ring finger domain with E2F transcriptional factors, cyclins/cdk complexes suggesting a function for this domain in mediating protein-protein interaction and a role for BRCA1 in cell cycle regulation (20). Recently, two other groups (29,30) have identified two BRCA1 splice variants BRCA1Δ672-4092 and BRCA1Δ11b. The molecular weight of p110 kDa BRCA1a protein is comparable to the 110 kDa BRCA1Δ11b (30). Since only the carboxy-terminal region of BRCA1 protein was previously tested and shown to function as a transcriptional activation domain (7,8), we investigated whether full length and various deletion mutants of splice variants BRCA1a and BRCA1b are able to activate transcription (20). Here we report for the first time that the amino-terminal region of BRCA1a but not BRCA1b can function as a transactivation domain when fused to a heterologous GAL4 DNA binding domain. Thus, BRCA1a contains two autonomous transcriptional activation domains, one at the amino-terminal region called BNT and the other at the carboxy-terminal region called BCT. BRCA1b has lost part of the amino-terminal trans-activation domain as a result of alternative splicing. Our results also suggest the presence of a negative regulatory domain at the carboxy-terminal regions of BRCA1 and BRCA1a proteins. It may be possible that mutations in the zinc finger domain found in patients with breast and ovarian cancer impair this activity, suggesting that loss of transcriptional activation by BRCA1 may lead to the development of breast and ovarian cancers. Alternately BRCA1b may function as a dominant-negative regulator of the transcriptional activation function of BRCA1/BRCA1a proteins.

Materials and methods

Mammalian expression plasmids. Full length and various deletions of the BRCA1a and BRCA1b coding sequences were fused in-frame with the GAL4 DNA binding domain (residues 1-147) in the GAL4 DNA binding domain vector (31) (a gift of P. Chambon). The CAT reporter plasmid 17MX2-tk-CAT contains two GAL4 binding sites linked to the CAT gene was also obtained from P. Chambon. The pCH110 plasmid containing the ß-galactosidase gene was used as an internal control to normalize the transfection efficiency.

Tissue culture and transient transfection. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated at $5x10^5$ per 100 mm dish and transfected by the calcium phosphate co-precipitation method (Promega) as described by the manufacturer with a total of 20 μg DNA (3 μg of GAL-4 DNA binding domain vector plasmid or GAL-4 BRCA1a/1b plasmids, 4 μg of 17MX2-tk-CAT reporter plasmid, 5 μg of pCH110 plasmid and 8 μg of Gem3 carrier plasmid). Cells were harvested at 48 h-63 h post-transfection. They were then lysed and the extracts assayed for ß-galastondase and CAT activities as described (18). The CAT assays were quantitated using a Fuji PhosphoImager. The experiments were repeated at least six times.

Results and Discussion

Full length cDNAs and several amino and carboxy-terminal deletions of BRCA1a and BRCA1b were subcloned in-frame with the GAL4 DNA binding domain vector obtained from P. Chambon (31). The GAL4 fusion constructs (Fig. 1) were co-transfected into NIH3T3 fibroblast cells, along with the reporter plasmid 17MX2-tk-CAT that contains two GAL4 binding sites linked to the chloramphenicol acelyltransferase gene (CAT) and an internal control plasmid CH110. Both full-length GAL4-BRCA1a and GAL4-BRCA1b plasmids failed to show CAT activity (Fig. 2) when compared to the GAL4 vector control. Two carboxy-terminal truncated BRCA1a plasmids, one lacking exons 21-24 and the other lacking exons 16-24, failed to show transactivation. Further carboxy-terminal deletion of BRCA1a (deletion of aa 263-1863) but not BRCA1b plasmid (deletions of aa 263-1863) showed ~7-10 fold CAT activity. These results suggest the presence of a transactivation domain at the amino-terminal region of BRCA1a (BNT) and an inhibitory domain (BID) at the carboxy-terminal region of the BRCA1a protein (Fig. 1). However, further carboxy-terminal deletion of exons 9 and 10 of BRCA1a and exons 9 and 10 alone when fused to the GAL4 DNA binding domain failed to show significant CAT activity. The fact that exons 9 and 10 as such failed to show activity indicates that additional sequences N-terminal and C-terminal to this region are also required for transcriptional activation. All these results suggest that apart from the carboxy-terminal region of BRCA1, the amino-terminal region of BRCA1a/BRCA1 (aa 1-262), which also includes the BRCA1 zinc finger domain, can also function as a transcription activation domain (BNT). The scenario we observe with BRCA1a/1b proteins parallel what is observed in Elk-1 and ΔElk-1 proteins where SRF binds to the ETA-1 domain of the Elk-1 protein thereby unmasking the ETA-2 domain facilitating phosphorylation and activation of transcription (32-35), in tumor suppressor gene p53 where MDM2 inhibits p53 transactivation by concealing its activation domain (36) and in yeast where GAL80 may inhibit GAL4 function by a similar phenomenon (37,38). Based on our results (this paper) and results of others (7,8, Rao *et al*, unpublished data), we have proposed a working hypothetical model as to how BNT and BCT transcriptional activation domains of BRCA1 might function *in vivo* (Fig. 3). In short, the BNT domain of BRCA1 protein can function *in vivo* independent of BCT, since truncation of the BCT domain results in significant transcriptional activation. The BCT domain may function either in the absence of BNT (as seen in BRCA1b protein) or in the presence of protein factors. It may be possible that such

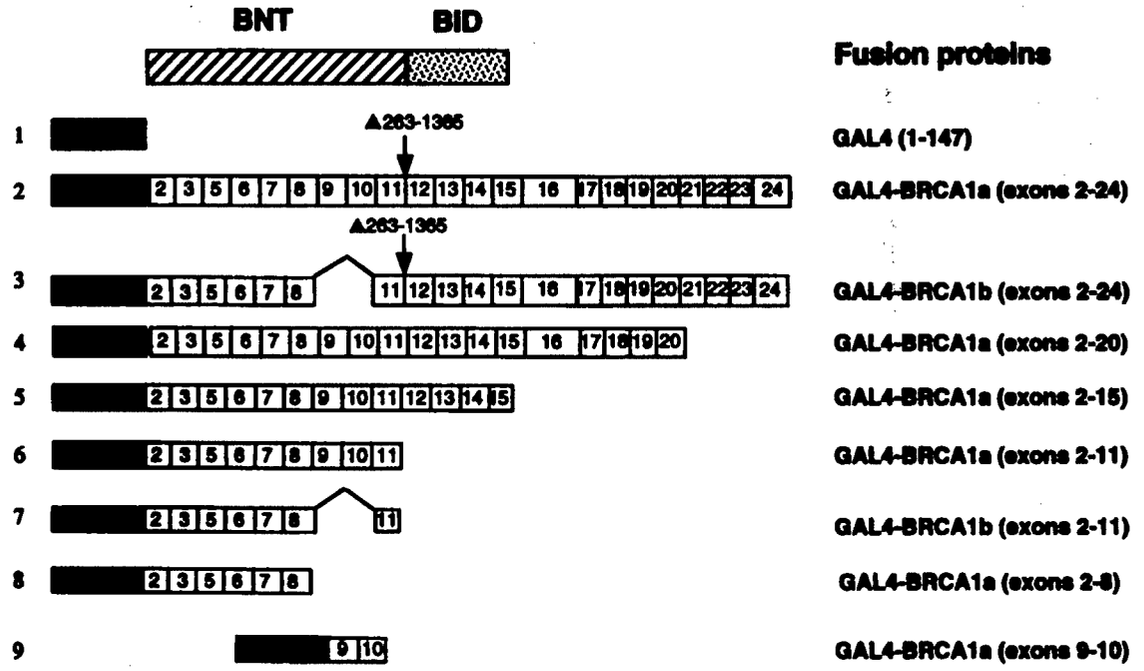


Figure 1. Transcriptional activation by the N-terminus of BRCA1 and BRCA1a proteins. Full length and various portions of the BRCA1a and BRCA1b coding regions were fused in-frame with the GAL4 DNA binding domain (residues 1-147). BNT, BRCA1 N-terminal activation domain; BID, BRCA1 inhibitory domain. The numbers above the arrows show the amino-acids that are deleted in BRCA1a/b and their derivatives. The amino-acids are numbered according to gene bank BRCA1 sequence (3) (#U14680).

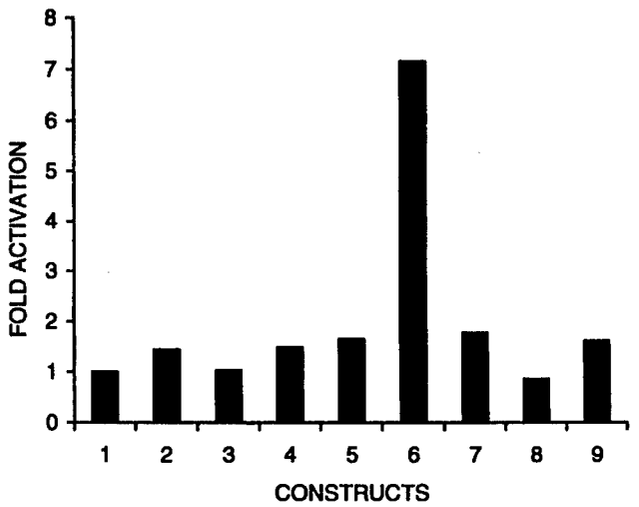
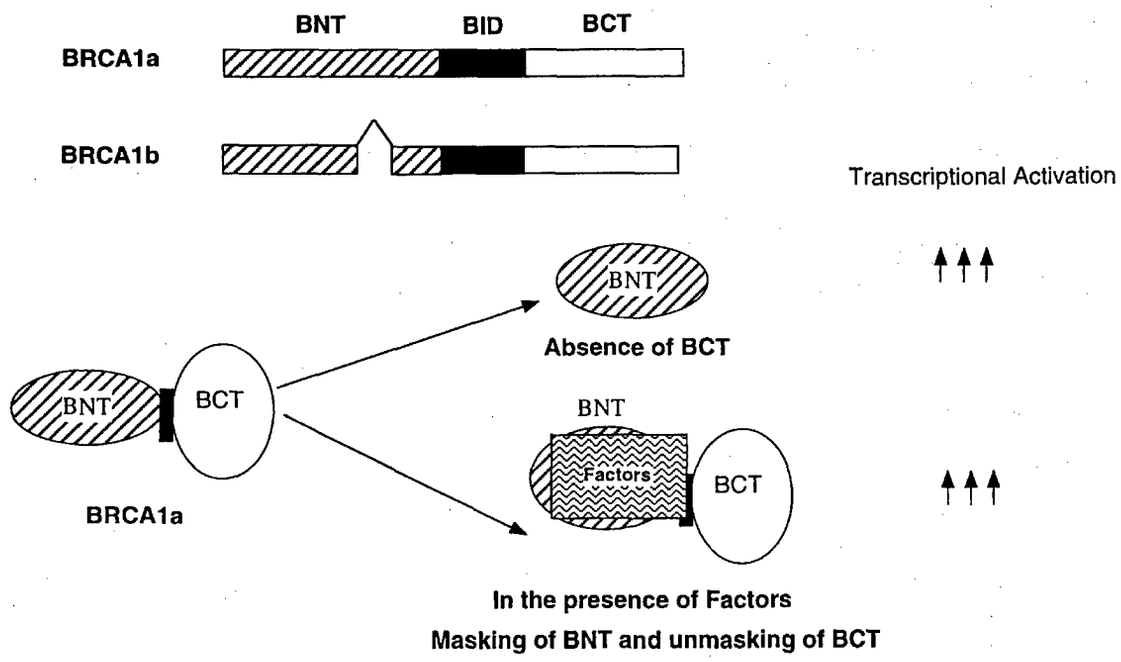


Figure 2. The constructs were co-transfected with the reporter construct 17MX2-tk-CAT into NIH3T3 cells and CAT activity was measured at 48 h-63 h following transfection. The numbers represent the plasmids shown in Fig. 1. The results are an average of at least six independent experiments. The CAT activity shown represents fold activation compared with control GAL4 DNA-binding domain vector alone.

factor(s) might bind to the BNT domain of BRCA1a protein, somehow conceal its activation domain and in the process unmask the BCT domain facilitating activation of transcription. The BNT domain may be functioning as a negative regulatory transcription activation domain for BCT. Although earlier results have indicated that the carboxy-terminal segment of BRCA1 associates with the RNA pol II holoenzyme (39), it may be possible that the amino-terminal transactivation domain (BNT) could modulate this interaction. Furthermore, our recent results indicate that both BRCA1a and BRCA1b proteins interact *in vitro* and *in vivo* with CBP co-activator (Cui *et al*, unpublished data). The binding of BRCA1a to CBP is weak when compared to BRCA1b suggesting that the BNT domain of BRCA1a may be functioning as an inhibitory domain for binding to CBP protein. Future experiments will be designed to address these issues. Previously, the zinc finger domain of BRCA1 was shown to interact both *in vitro* and *in vivo* with BARD1 (24) and *in vitro* with E2F transcriptional factors and cyclins/cdk complexes (20), which suggested a function for this domain in mediating protein-protein interactions and a role for BRCA1 in cell cycle regulation. The BRCA1 zinc finger domain is the location of some of the most frequently occurring mutations linked to breast and ovarian cancers. One BRCA1 mutation contains a frame shift in exon 2 (188 del 111) that removes the zinc finger domain from the protein. The 185 del AG mutation, which is the most common BRCA1 mutation [about 1% of Ashkenazi Jewish individuals (40)], disrupts the BRCA1 gene product at the first residue of the C_3HC_4 domain. Some frequent missense mutations Cys61, Gly, Cys 64, Gly and Cys 64 Tyr also disrupt the zinc finger domain structure. It may be possible that loss of transcriptional activation or protein-protein interactions in patients with mutations in the zinc finger domain could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast cancer.



BNT **BRCA1 Amino-terminal Transcriptional activation domain**

BCT **BRCA1 Carboxy-terminal Transcriptional activation domain**

BID **BRCA1 Inhibitory Domain**

Figure 3. Hypothetical model as to how the two transcriptional activation domains BNT and BCT of the BRCA1 proteins might function *in vivo*.

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BRCA1 splice variants BRCA1a and BRCA1b associate with CBP co-activator

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Abstract. The tumor suppressor gene BRCA1, is a nuclear phosphoprotein which associates with RNA polymerase II holoenzyme. CBP is a component of the holoenzyme. Previously, we have characterized two new BRCA1 splice variants BRCA1a/p110 and BRCA1b/p100. In the present study, the carboxy-terminal domain of transcription factor CBP interacts both *in vivo* and *in vitro* with full length BRCA1a and BRCA1b proteins as demonstrated by mammalian two-hybrid assays, co-immunoprecipitation/Western blot studies, GST binding assays and histone acetyl transferase (HAT) assays of BRCA1 immunoprecipitates from human breast cancer cells. Our results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP associated HAT/FAT (transcription factor acetyl-transferase) activity for acetylation of either themselves or general transcription factors or both to specific promoters resulting in transcriptional activation.

Introduction

BRCA1, a familial breast and ovarian cancer susceptibility gene is mutated in 45% of the inherited breast and ovarian cancers and 90% of families with high incidence of both breast and ovarian cancers (1-3). The BRCA1 cDNA codes for a 1863 amino acid protein with an amino-terminal zinc finger domain and a carboxy-terminal acidic region typical of several transcriptional factors. Recently, the carboxy-terminal region of BRCA1 was shown to activate transcription (4,5, Rao, unpublished data) when fused to a GAL4 DNA binding domain. Our results suggest the presence of an autonomous transcriptional activation domain at the amino-terminal region of BRCA1 proteins (Cui JQ and Rao VN, unpublished data) similar to BRCA2 (6). Earlier results indicated that intact BRCA1 associates with the RNA polymerase II holoenzyme and the carboxy-terminal region of BRCA1 is important for holoenzyme binding (7). The BRCA1 protein is a nuclear phosphoprotein (8-12) which localizes to nuclear dots during S phase (10,13,14) and associates with Rad51 and BARD1 suggesting a role for BRCA1 in DNA repair and the maintenance of genome stability (14,15). Recently DNA damaging agents were shown to alter the subcellular localization and phosphorylation of p220 BRCA1 protein suggesting a role in S phase DNA damage dependent cell cycle check point response (16). Our results suggest that BRCA1 proteins accumulate in the nucleus in the absence of serum and in the cytoplasm in the presence of serum in some cells revealing a new type of regulation that may modulate the activity of BRCA1 gene (11). Several other functions such as growth and tumor suppression (9,17,18), induction of apoptosis (19) have been associated with BRCA1 proteins indicating BRCA1 to be truly a multifunctional protein. Previously, we have isolated and characterized two new BRCA1 splice variants, BRCA1a (p110) and BRCA1b (p100) (11). BRCA1b protein has lost part of the potential amino-terminal transcriptional activation domain as a result of alternative splicing (Cui JQ and Rao VN, unpublished data). BRCA1a and BRCA1b proteins are phosphoproteins containing phosphotyrosine that are localized both in the cytoplasm and nucleus and associate with E2F transcriptional factors, cyclins/cdk complexes suggesting a role for BRCA1 proteins in cell-cycle regulation (11).

Materials and methods

GST pull down assay. GST-CBP1 (residues 461-662) and GST-CBP2 (residues 1621-1877) were expressed and purified from *Escherichia coli* as reported previously (11). For *in vitro* binding assay ^{35}S-methionine labeled *in vitro* translated full length BRCA1a and BRCA1b (Rao, unpublished data) proteins were diluted in TNN buffer (20) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 µl/ml leupeptin, 3% aprotinin and 1 mM sodium orthovanadate. The solutions were precleared with GSH-beads for 1 h and then incubated with GST-CBP1 beads or GST-CBP2 beads for 2 h at 4°C. The beads were then washed five times in TNN buffer and heated

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Abbreviations: CBP, CREB-binding protein; HAT, histone acetyl transferase; GST, glutathione S-transferase; FAT, transcription factor acetyl-transferase

Key words: BRCA1, CBP co-activator, histone acetyl transferase, transcription factor acetyl-transferase

in SDS sample buffer and loaded on a 10% SDS PAGE. The gels were fixed, treated with enhance, dried and exposed to X-ray films or exposed and scanned using a Fuji BioImaging analyzer.

IP-HAT assays. CAL51 cells were cultured in 75 cm (2) culture flasks in DMEM medium containing 10% FBS, 0.6 μg/ml bovine insulin, $5x10^{-3}$ μg/ml transferrin, 1% penicillin-streptomycin. IP-HAT assay was done essentially as described (21) except with slight modifications. Briefly, cell pellets were collected by trypsinization and washed once in PBS. The cells were lysed in 1 ml of lysis buffer IPH (21) except that IGEPAL CA-630 (Sigma) was substituted for NP-40. The lysis mixture was then incubated on ice for 20 min and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Either CBP A-22 (Santa Cruz Biotechnology) or BRCA1 D-20 (Santa Cruz Biotechnology) or BRCA1a recombinant polyclonal antibody or Myc polyclonal antibody (Santa Cruz Biotechnology) were added to 1 ml of extract and incubated at 4°C for 2 h. A 1:1 mixture (15 μl total) of protein A-sepharose/protein G-sepharose was added and the mixture rotated overnight at 4°C. The immunocomplexes were centrifuged and washed three times with 1 ml lysis buffer IPH. 1.25 μl of 20 mg/ml histone or BSA, 1 μl of ^{3}H-acetyl Co A (4.3 Ci/mMol Amersham) were added to the immunocomplexes to a total volume of 30 μl with lysis buffer IPH. HAT assays were performed at 30°C for 45 min to 1 h. The reactions were spotted onto Whatman P-81 filters, washed five times with 0.2 M Na_2Co_3 15 min each, dried and counted in a Beckman liquid scintillation counter.

Immunoprecipitation/Western blot assay. CAL51 cells ($1x10^8$) were collected by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in 1.0 ml RIPA buffer and the lysate was centrifuged at 14,000 rpm for 30 min at 4°C. To the supernatant normal rabbit IgG and protein A-sepharose CL-4B (Pharmacia) was added and rotated gently at 4°C overnight. The supernatant was then incubated with BRCA1 polyclonal antibody (Santa Cruz SC-641) or CBP polyclonal antibody (Santa Cruz SC-369) for an hour at 4°C. The proteins were then crosslinked to protein A-sepharose CL-4B for 2 h at 4°C. The beads were then washed twice with RIPA buffer and 1/10 detergent buffer [150 mM NaCl, 40 mM Tris-HCl (pH 7.5), 0.1% NP40, 0.01% sodium deoxycholate, 0.01% SDS, 1 mM EDTA, 1 mM EGTA]. The beads were boiled with 40 μl of SDS-PAGE loading buffer for 5 min and electrophoresed either on a 6% SDS-PAGE (for CBP analysis) or 10% SDS-PAGE (for BRCA1 analysis) and subjected to Western blot analysis as described previously (9). Following incubation of the membranes with CBP or BRCA1 antibodies, the proteins were detected using a Western exposure chemiluminescent detection system from ECL as suggested by the manufacturer (Amersham).

Plasmid construction and transfections. Full length BRCA1a and BRCA1b cDNAs (Rao, unpublished data) were subcloned in frame with the GAL4 DNA binding domain vector obtained from P. Chambon (22). PHK Gal4-FOS, pHK3n CBP1 VP16 (expressing CBP 461-662 in-frame with the VP16AD), pHK3n CBP2 VP16 (expressing CBP 1621-1877 in-frame with the VP16 (AD) G5E1bCAT and pHK3nVP16 plasmids were a gift from T. Kouzarides. PSGVP16 (expressing the VP16 activation domain) plasmid has been described previously (33). U20S human osteosarcoma cells were maintained in McCoy's 5A supplemented with 10% FCS and grown at 37°C (5% CO_2). These cells were transfected using the calcium phosphate co-precipitation method (Promega) as described previously (9). At 48 h post transfection cell extracts were prepared from transfected cells and subjected to ß-galactosidase and CAT assays. The CAT assays were quantitated with a Fuji PhosphoImager. The experiments were repeated at least 5-6 times. For studying the ectopic expression of GAL4 BRCA1a and GAL4 BRCA1b fusion proteins, COS-7 cells were transfected with 20 μg of GAL4 DNA binding domain vector DNA or 20 g of GAL4 BRCA1a DNA or GAL4 BRCA1b DNA using the calcium phosphate co-precipitation method as described above. At 48-56 h post transfection cell extracts were prepared and ~90 μg of protein was loaded on an 8% SDS PAGE and subjected to Western blot analysis using anti GAL4 (DBD) polyclonal antibody (Sant Cruz Biotechnology, Inc.).

Results and Discussion

Recently, BRCA1 protein was shown to associate and co-purify with RNA polymerase II holoenzyme (7). Since CREB binding protein (CBP) is a component of the holoenzyme (23), we speculated whether BRCA1 could interact directly with co-activator CBP which would bring the holoenzyme into play. In the present study, both full length BRCA1a and BRCA1b proteins interact with the carboxy-terminal domain of CBP protein (1620-1877) as demonstrated by mammalian two-hybrid assays. Direct physical interaction of CBP with BRCA1a/BRCA1b proteins were shown by glutathione S-transferase fusion protein binding assays, co-immunoprecipitation/Western blot studies and HAT activity assays of BRCA1 immunoprecipitates from breast cancer cells. All these results suggest that BRCA1 proteins interact with CBP and targeting CBP-associated HAT/FAT activity to specific promoters may be one of the mechanisms by which BRCA1 proteins function.

CBP is a well characterized co-activator which is a component of the RNA polymerase II holoenzyme (23). CBP interacts with the phosphorylated form of transcription factor CREB and activates transcription (24). A closely related co-factor p300 was identified on the basis of its interaction with adenovirus E1A (25). Both CBP and p300 interact with a variety of cellular transcriptional activators that include nuclear hormone receptors, CREB, c-Jun/v-Jun, c-Myb/v-Myb, stat-1, stat-2, c-*fos*, Myo D, NFkBp65, Elk-1, p53 and basal components of the transcriptional apparatus (24,26-28).

To demonstrate interaction between CBP and BRCA1a/BRCA1b proteins *in vitro* and *in vivo*, we have used three different approaches: GST fusion protein pull down assays, a mammalian two-hybrid system and immunoprecipitation/Western blotting method. The CBP protein has at least two domains which bind to transcriptional factors: a region containing residues 461-662 (designated CBP1) which is required for binding CREB, c-Jun, cMyb; a region containing residues 1621-1877 (designated CBP2) which is required for interactions with E1A, c-Fos and TF11B (24,26). GST fusion proteins containing CBP1 and CBP2 domains were expressed as described (11). To examine whether BRCA1a/1b proteins

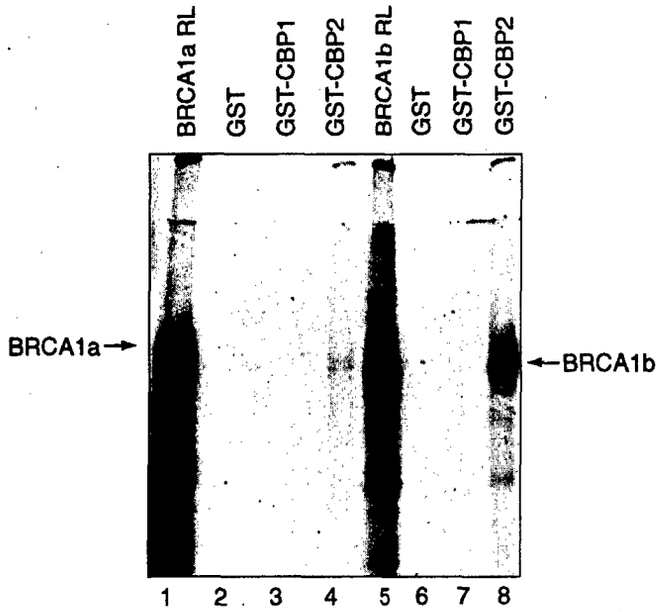
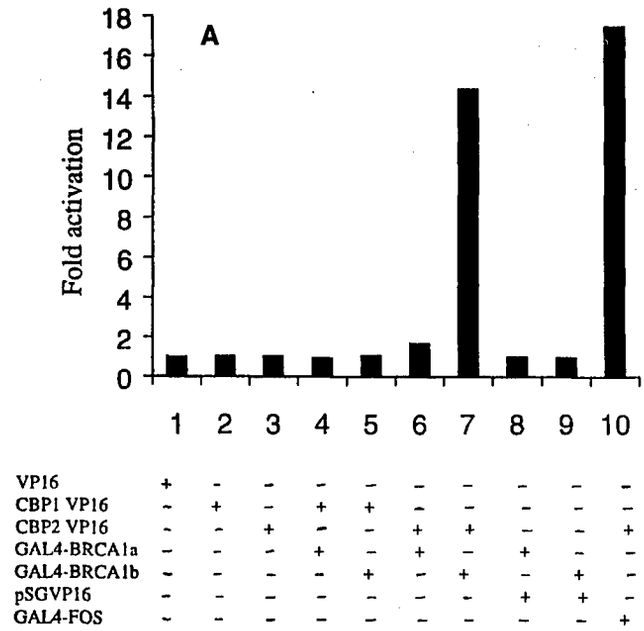
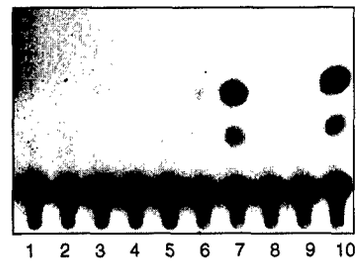


Figure 1. Direct physical interactions of CBP with BRCA1a and BRCA1b *in vitro*. GST, GST-CBP1 or GST-CBP2 were incubated with *in vitro* translated ^{35}S-labeled BRCA1a or BRCA1b and subjected to GST pull down assay. 1 µl of the *in vitro* translated BRCA1a and BRCA1b proteins were run as controls. Arrows represent the *in vitro* translated BRCA1a (p110) and BRCA1b (p100) proteins.





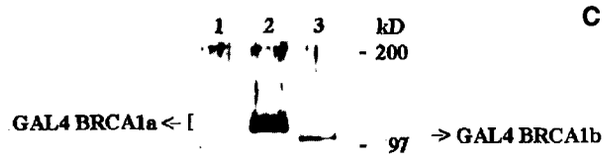


Figure 2. Full length BRCA1a and BRCA1b interact with the carboxy-terminal domain of CBP (CBP2) in a mammalian two-hybrid system. A, U20S human osteosarcoma cells were co-transfected with 4 µg pG5E1BCAT reporter plasmid, 1 µl of VP16 expression plasmids (pHK3nVP16 or pSGVP16 or pHK3nCBP1VP16 or pHK3nCBP2VP16) and 1 µl of GAL-4 expression plasmids (GAL-4 BRCA1a or GAL-4 BRCA1b or GAL-4 Fos). Total DNA was kept constant at 20 µg. The CAT activity shown represents fold activation compared with the VP16 vector alone. The activity of the vector is normalized to a value of 1. B, The numbers represent the columns shown in A. Data that was used to calculate the fold activity represented in A. The experiments were repeated at least six times. C, Ectopic expression of BRCA1a and BRCA1b in COS cells. Extracts from control cells (lane 1), GAL4 BRCA1a-transfected cells (lane 2) or GAL4 BRCA1b-transfected cells (lane 3) were subjected to 8% SDS-PAGE and Western blot analysis with anti-GAL4 (DBD) polyclonal antibody. The doublet bands seen in lane 2 represent phosphorylated forms of GAL4 BRCA1a fusion proteins and the band in lane 3 represents GAL4 BRCA1b fusion protein.

directly interact with CBP, *in vitro* binding was performed with *in vitro* translated BRCA1a/1b and GST-CBP proteins. *In vitro* transcription and translation of BRCA1a and BRCA1b in the presence of [^{35}S]-methionine generated radiolabeled BRCA1a and BRCA1b polypeptides of 110 and 100 kDa respectively (Fig. 1, lanes 1 and 5). These radio-labeled proteins were passed through GST-CBP1, GST-CBP2 and a GST control column. Both BRCA1a and BRCA1b were found to bind to GST-CBP2. As no BRCA1a and BRCA1b were detected in the eluates from the GST, GST-CBP1 columns (Fig. 1, lanes 2, 3, 6 and 7), these results suggest that both BRCA1a/BRCA1b proteins bind specifically to GST-CBP2. The BRCA1a splice variant binds weakly to GST-CBP2 compared to BRCA1b similar to what was observed earlier with binding to E2F and cyclins (11). It may be possible that the amino-terminal transcriptional activation domain present in BRCA1a may be serving as an inhibitory domain for binding to CBP protein. Taken together these results suggest that BRCA1a/1b interact physically with CBP, since GST-CBP2 binds to *in vitro* translated BRCA1a/1b proteins.

Having established that the CBP2 domain of CBP can directly associate with intact BRCA1a and BRCA1b proteins *in vitro*, we next studied the interaction *in vivo* using the mammalian two-hybrid system. We subcloned the full length BRCA1a and BRCA1b cDNA's in frame with the GAL4 DNA binding domain vector (22). The CBP1 VP16 and CBP2 VP16 plasmids in which the CBP1 and CBP2 domains have been fused to the activation domains of VP16 were obtained from T. Kouzarides (26). U20S human osteosarcoma cells or COS cells were co-transfected with CBP1 VP16 or CBP2 VP16 and GAL4 BRCA1a or GAL4 BRCA1b and G5E1bCAT as reporter. Fig. 2A shows that in the two hybrid *in vivo* interaction experiments, the CBP2VP16 protein simulates GAL4 BRCA1b activity specifically (15 fold). This activation is not seen with the CBP1VP16 hybrid protein nor with the pSGVP16 vector alone (Fig. 2A and B). The stimulation observed with GAL4 BRCA1b was comparable to that seen with GAL4-Fos and CBP2 VP16 (~17 fold). In the same experiment, GAL4 BRCA1a showed marginal stimulation in

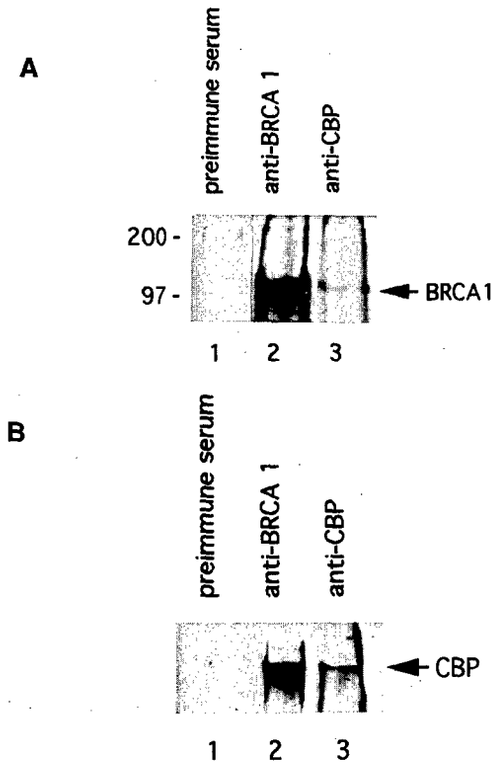


Figure 3. *In vivo* interaction of CBP with BRCA1 proteins in CAL51 cells. A, Western blot analysis of CAL51 whole cell extract with anti-BRCA1 after immunoprecipitation with preimmune serum (lane 1), anti-BRCA1 (lane 2) and anti-CBP (lane 3). B, Immunoblot with anti-CBP after immunoprecipitation of CAL51 whole cell extract with preimmune serum (lane 1), anti-BRCA1 (lane 2) and anti-CBP (lane 3). The BRCA1 arrow in A (lane 3) represents BRCA1b (p100).

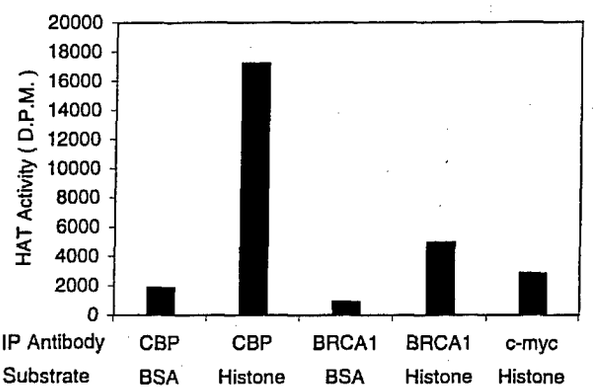


Figure 4. BRCA1 antibody immunoprecipitates HAT activity in an IP-HAT assay. CAL51 whole-cell extracts were immunoprecipitated with CBP or BRCA1 or Myc antibodies as indicated. The Myc antibody was used as a negative control (21). The immune complexes were washed extensively and assayed for their ability to acetylate BSA or histones.

presence of CBP2 VP16 (Fig. 2A and B), consistent with the *in vitro* binding results (Fig. 1). We studied the expression of GAL4 BRCA1a fusion protein in transiently transfected COS cells by Western blot analysis using a GAL4 DNA binding domain specific polyclonal antibody. The GAL4 BRCA1a protein was expressed at higher levels compared to GAL4 BRCA1b fusion protein (Fig. 2C) thus ruling out the possibility that the weak interaction observed *in vivo* with VP16 CBP2 is due to inappropriate expression of BRCA1a protein. It may be possible that the amino-terminal transcriptional activation domain present in BRCA1a may be serving as an inhibitory domain for binding to CBP protein.

Interactions between CBP and BRCA1 proteins *in vivo* were further demonstrated by using immunoprecipitation/immunoblot analysis. To study these associations in a physiologically relevant environment, cell extracts from human breast cancer cells CAL-51 were immunoprecipitated with anti-CBP or anti-BRCA1 antibodies and then Western blotted with anti-BRCA1 or anti-CBP antisera. The anti-BRCA1 antiserum has been used previously to detect BRCA1a/1b splice variants by immunoprecipitation and Western blot analysis (9,11,19). The CAL-51 cell lysates immunoprecipitated with anti-CBP antisera showed an immunoreactive band at 100 kDa that was recognized with anti-BRCA1 antisera (Fig. 3A, lanes 2 and 3). In a reciprocal experiment, immunoprecipitation of CAL-51 cell lysates with anti-BRCA1 antibody showed an immunoreactive band that was recognized by antibodies against CBP (Fig. 3B, lanes 2 and 3). These results suggest that an association between BRCA1b and CBP occurs in intact cells. The precise mechanism by which transcription factors stimulate the transcriptional machinery through contacting CBP/p300 is unclear. Recently, p300/CBP and P/CAF were shown to possess histone acetyl transferase activities (21,29,30) suggesting that recruiting CBP/p300 associated HAT activity to specific promoters may be one of the mechanisms by which transcription factors function.

We next tested the BRCA1 immunoprecipitates from CAL-51 cells for HAT activity. Our results demonstrate that these immunocomplexes show HAT activity that is specific for histones (Fig. 4). Antibodies against nuclear proteins like Myc only precipitated a background non-specific activity (Fig. 4). The HAT activity associated with the BRCA1 immunoprecipates can either be due to the acetylase activity complexed specifically with CBP (21) or there could be a possibility that BRCA1 proteins could themselves possess intrinsic HAT activity similar to Gcn5, CBP/p300 and TAF250 (21,26,29-32). In summary, our results demonstrate the binding of BRCA1 proteins to CBP and suggest that BRCA1 may carry HAT activity when associated with CBP similar to E1A protein (21). The question whether the function(s) (transactivation, protein-protein interactions, etc.) of BRCA1 proteins can be modified by acetylation similar to that observed for tumor suppressor gene p53 and whether BRCA1 proteins possess intrinsic HAT activity awaits further experimentation (30). The binding of BRCA1a to CBP is weak or almost negligible when compared to BRCA1b indicating that the amino terminal transactivation domains present in BRCA1a protein (Rao, unpublished data) may inhibit the binding to CBP. Furthermore, based on these results, we can speculate that the levels of BRCA1a/1b proteins may be critical for identifying individuals with greater risk for developing breast cancer. Taken together these results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of acetyl transferase containing CBP co-activators for acetylation of either themselves or general transcription factors or both to specific promoters resulting in transcriptional activation. Alternatively, BRCA1 proteins may also serve as adaptor proteins which could bridge the association between CBP co-activator and RNA polymerase II complexes. It may

be possible that lack or impaired binding of disrupted BRCA1 proteins or variant BRCA1 proteins to CBP in patients with mutations could deprive the cell of an important mechanism for regulating cell proliferation leading to the development of breast and ovarian cancers.

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SHORT REPORT

The BRCA2 is a histone acetyltransferase

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Patients carrying mutations in BRCA1 or BRCA2 tumor suppressor genes have shown to have high risk in developing breast and ovarian cancers. Two potential functions of BRCA2 were proposed which includes role in the regulation of transcription and also in DNA repair. Forty-five-amino acid region encoded by exon 3 of BRCA2 was shown to have transcriptional activation function. Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation. Since BRCA2 appear to function as a transcriptional factor, we have tested for Histone acetyl transferase (HAT) activity of BRCA2. Here, we present evidence that BRCA2 has intrinsic HAT activity, which maps to the amino-terminal region of BRCA2. Our results demonstrate that BRCA2 proteins acetylate primarily H3 and H4 of free histones. These observations suggest that HAT activity of BRCA2 may play an important role in the regulation of transcription and tumor suppressor function.

Keywords: BRCA2; histone acetyl transferase; protein-protein interaction; tumor suppressor

Alterations in *BRCA1* and *BRCA2* tumor suppressor genes have been shown to be involved in 90% of familial breast cancers (Newman *et al.*, 1988; Miki *et al.*, 1994; Easton *et al.*, 1993; Wooster *et al.*, 1994; Wooster and Stratton, 1995). Recent studies revealed that both BRCA1 and BRCA2 are involved in ovarian and prostate cancers. Interestingly, BRCA2 was found to be more associated with male breast cancer compared to BRCA1 (Wooster *et al.*, 1994). Patients with BRCA2 mutations were also found to be at a higher risk with a variety of other cancers including carcinomas of pancreas, prostate and colon (Thorlacius *et al.*, 1996; Phelan *et al.*, 1996; Gudmundsson *et al.*, 1995; Tonin *et al.*, 1995). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (Wooster *et al.*, 1995; Bork *et al.*, 1996). BRCA2 and BRCA1 proteins have been shown to interact with Rad 51 which suggests that they play a role in DNA repair (Scully *et al.*, 1997; Sharan *et al.*, 1997; Zhang *et al.*, 1998). BRCA1 was also shown to induce apoptosis suggesting that BRCA proteins may play a role in the regulation of apoptosis of cells (Shao *et al.*, 1996; Rao *et al.*, 1996). It remains to be seen whether BRCA2 plays a similar role in apoptosis.

Interestingly, both *BRCA1* and *BRCA2* gene products are regulated in a cell cycle-dependent manner and have a potential transactivation function (Rajan *et al.*, 1996; Vaughn *et al.*, 1996; Chapman and Verma, 1996; Monteriro *et al.*, 1996; Milner *et al.*, 1997; Wang *et al.*, 1997; Cui *et al.*, 1998a). Recently, we have shown that BRCA1 proteins interact with transcriptional co-activator CBP suggesting that BRCA1 has a role in the regulation of transcription (Cui *et al.*, 1998b). Exon 3 of BRCA2 was found to have weak homology with transcriptional factor c-*jun* and also shown to activate transcription in mammalian cells (Milner *et al.*, 1997). These results suggest that BRCA2r has a role in the regulation of gene expression.

Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation (Brownell *et al.*, 1996; Parthun *et al.*, 1996; Yang *et al.*, 1996; Orgyzko *et al.*, 1996; Mizzen *et al.*, 1996; Roth and Allis, 1996; Wade and Wolffe, 1997; Pazin and Kadonaga, 1997; Wolffe, 1997). This view is supported by the identification of Histone acetyl transferase (HAT) activity associated with several transcription factors including p300/CBP, GCN5-related factors, p/CAF, SRC-1 and TAF_{II} 250. These results suggest that some transcriptional activators operate by disrupting the nucleosomal structure through acetylation of histones leading to the activation of gene expression.

Here, we report for the first time that the amino-terminal region of BRCA2 has intrinsic HAT activity from which it may be inferred that BRCA2 joins the above list of transcriptional activators/factors that possess HAT activity. This intrinsic BRCA2-HAT activity may play a key role in the tumor suppressor function of BRCA2.

Recently, we have cloned an alternatively spliced isoform, BRCA2a. This variant BRCA2a lacks a transcriptional activation domain (exon 3) as a result of alternative splicing (our unpublished results). In order to test the HAT activity of BRCA2, we have expressed the amino-terminal region of BRCA2 (aa 1–500) and its isoform BRCA2a (aa (1–18)-(105–500)) as GST-fusion proteins in bacteria by cloning appropriate BRCA2 cDNA fragments into a GST expression vector (Our unpublished results). Purified recombinant proteins of BRCA2 and BRCA2a were assayed for histone acetyl transferase activity. Amino-terminal domains of both BRCA2 and BRCA2a clearly demonstrated histone acetyl transferase activity (Figure 1). Control samples where BRCA2 or BRCA2a was replaced with bovine serum albumin (BSA) showed no

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significant HAT activity. Similar control experiments where histones were replaced by BSA (lysine rich nonhistone protein) also failed to show significant acetyl transferase activity. This suggests that BRCA2 proteins show specific acetyl transferase activity to histones (Figure 1). Therefore, we conclude that BRCA2 *per se* is a histone acetyl transferase. Since the amino-terminal region of BRCA2 and BRCA2a show HAT activity, we conclude that the exon 3 (aa 18–105) responsible for the transactivation function of BRCA2 is not needed for HAT activity function. These results suggest that the transactivation and HAT functional domains of BRCA2 do not overlap with each other (Figure 2).

In order to determine which histones are acetylated by BRCA2 proteins, we have carried out HAT assay with free core histones and analysed the resulting products by SDS-polyacrylamide gel electrophoresis followed by fluorography. Our results demonstrate that BRCA2 proteins acetylated primarily H3 and H4 of free histones (Figure 3). We have also confirmed these results using individual free histones (data not shown).

In order to determine the HAT activity associated with BRCA2 *in vivo*, we have carried out immunoprecipitation HAT assay. Immunoprecipitation of BRCA2 from whole cell extracts was tested for acetyl transferase activity. Our results demonstrate that immunoprecipitated BRCA2 carries acetylase activity specific for histones (Figure 4). These *in vitro* and *in vivo* results support the conclusion that BRCA2 has intrinsic HAT activity. It is conceivable that as in the case of CBP/p300 (which shows intrinsic HAT activity), transcriptional activators recruit BRCA2 and utilize its intrinsic HAT activity for their transcriptional activation properties. It is also possible that BRCA2 also in turn recruits other factors (like p/CAF, p300/CBP) that possess distinct HAT activity and thereby disrupt the nucleosomal structure through their cooperative HAT activity. This results in the activation of gene expression interacts with CBP both *in vitro* and *in vivo*. Therefore, it is tempting to speculate that the target genes of BRCA2 play key roles in growth inhibition, differentiation and apoptosis. Identification of these genes may provide clues to the role of BRCA2 in neoplasia. Because of its large size, it is conceivable that BRCA2 has multicellular functions which include DNA repair, transcriptional activation, HAT etc. It is possible that BRCA2/Rad 51 Complex may use HAT activity to disrupt the nucleosomal structure to recognize

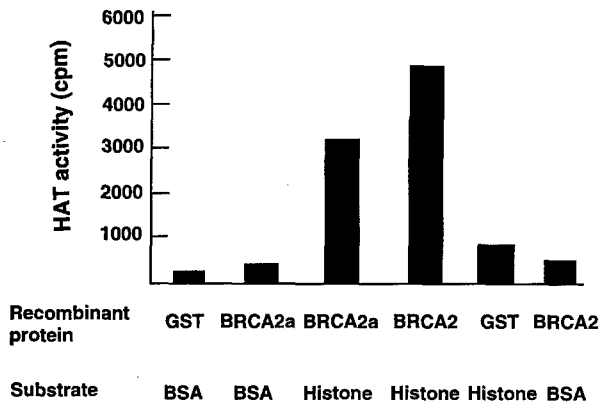

Figure 1 BRCA2 has intrinsic HAT activity. The amino-terminal region of BRCA2 (aa 1–500) and BRCA2a ((1–18)-(105–500)) were expressed as GST fusion proteins in bacteria and subsequently purified. HAT assays were caried out as described but with slight modification (Bannister and Kouzarides, 1996; Herrera *et al.*, 1997). Approximately 50–100 ng of GST-fusion proteins of BRCA2 and BRCA2a were used to acetylate 15 μg of core histones (Boehringer Mannheim) in the presence of [^{3}H]acetyl CoA

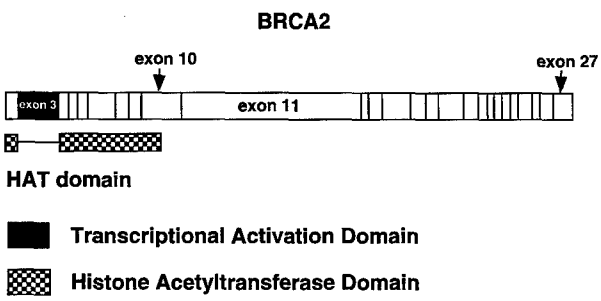

Figure 2 Schematic representation of the functional domains of BRCA2

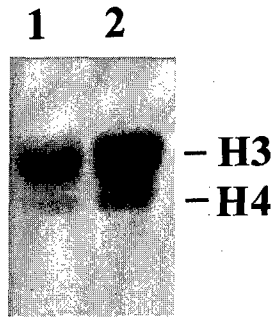

Figure 3 Acetylation profile and Substrate specificity of BRCA2 and BRCA2a. Recombinant BRCA2a (aa 1–500) (lane 1) and BRCA 2 ((1–18)-(105–500)) (lane 2) were incubated with core histones as described above. [^{14}C]acetylated histones were separated on SDS–PAGE and detected by autoradiography

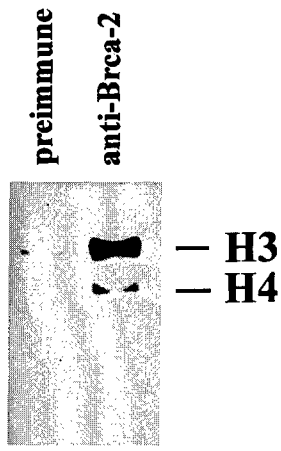

Figure 4 BRCA2 antibodies immunoprecipitate HAT activity. Immunoprecipitations (IP) were performed from NIH3T3 whole cell extract with either anti-BRCA2 (Santa Cruz) antibody or pre-immune serum. These IPs were tested for their ability to acetylate free histones as described above. Pre-immune serum served as a negative control

damaged DNA for DNA repair. Patients with mutations in HAT and/or transactivation domains of BRCA2 may show a loss of gene expression whch are critical for growth inhibition and differentiation and result in a subset of familial breast and prostate cancers. One can use BRCA2-HAT assay for screening patients with BRCA2 mutations.

Acknowledgements
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SHORT REPORT

The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21$^{WAF1/CIP1}$ promoter

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Inherited mutations in the breast and ovarian cancer susceptibility gene BRCA1 are associated with high risk for developing breast and ovarian cancers. Several studies link BRCA1 to transcriptional regulation, DNA repair, apoptosis and growth/tumor suppression. BRCA1 associates with p53 and stimulates transcription in both p53 dependent and p53-independent manners. BRCA1 splice variants BRCA1a (p110) and BRCA1b (p100) associates with CBP/p300 co-activators. Here we show that BRCA1a and BRCA1b proteins stimulate p53-dependent transcription from the p21$^{WAF1/CIP1}$promoter. In addition, the C-terminal second BRCA1 (BRCT) domain is sufficient for p53 mediated transactivation of the p21 promoter. Previous studies emphasized the importance of the BRCT domain, which shows homology with p53 binding protein (53BP1), in transcriptional activation, growth inhibition and tumor suppression. Our findings demonstrate an additional function for this domain in protein–protein interaction and co-activation of p53. We also found that BRCA1a and BRCA1b proteins interact with p53 *in vitro* and *in vivo*. The p53 interaction domain of BRCA1a/1b maps, *in vitro*, to the second BRCT domain (aa 1760–1863). The BRCT domain binds to the central domain of p53 which is required for sequence specific DNA binding. These results demonstrate for the first time the presence of a second p53 interaction domain in BRCA1 proteins and suggests that BRCA1a and BRCA1b proteins, like BRCA1, function as p53 co-activators. This BRCT domain also binds *in vitro* to CBP. These results suggest that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation.

Keywords: BRCA1; BRCA1a; BRCA1b; p53; p21$^{WAF1/CIP1}$; BRCT domain; CBP/p300

BRCA1, a familial breast and ovarian cancer susceptibility gene, is linked to 45% of the families with inherited breast cancers and about 90% of families with inherited breast and ovarian cancers (Miki *et al.*, 1994; Easton *et al.*, 1995; Ford *et al.*, 1995). BRCA1 encodes a protein of 1863 amino acids. The unique amino-terminal zinc finger domain interacts with BARD1 (Wu *et al.*, 1996), E2F transcriptional factors, cyclins and cyclin-dependent kinases (Wang *et al.*, 1997). Two, tandem carboxy-terminal BRCT domains (BRCA1 C-terminal domain (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997) are involved in transcription activation, growth inhibition and tumor suppression (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Rao *et al.*, unpublished results; Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997). The BRCT domains are targets for cancer associated mutations (Couch and Weber, 1996) and are conserved evolutionary (Szabo *et al.*, 1996). Several recent reports have shown moderate homology between the BRCT domains of BRCA1 and the C-terminal region of p53-binding protein (5BP1), the yeast RAD9 protein and multiple proteins involved in cell cycle checkpoint functions responsive to DNA damage (Koonin *et al.*, 1996; Bork *et al.*, 1997; Callebaut and Mornon, 1997). Recently, we and others showed that the N- and C-terminal regions of BRCA1, BRCA1a and BRCA1b proteins activate transcription when fused to GAL4 DNA binding domain (Cui *et al.*, 1998a; Chapman and Verma, 1996; Monteiro *et al.*, 1996), and associate with RNA polymerase II holoenzyme (Scully *et al.*, 1997a) and CBP/P-300 co-activator (Cui *et al.*, 1998b), suggesting a potential role for BRCT domains in the regulation of transcription. The multiple functions of BRCA1 protein(s) include growth/tumor suppression (Holt *et al.*, 1996; Thompson *et al.*, 1995; Rao *et al.*, 1996; Humphrey *et al.*, 1997), induction of apoptosis (Shao *et al.*, 1996), cell cycle regulated expression, DNA repair and the maintenance of genomic stability (Lane *et al.*, 1995; Marquis *et al.*, 1995; Scully *et al.*, 1997b; Brugarolas and Jacks, 1997). The BRCA1 protein physically associates, both *in vitro* and *in vivo*, with p53 tumor suppressor gene and stimulates p53-dependent transcription from the p21$^{WAF1/CIP1}$ and bax promoters (Ouchi *et al.*, 1998; Zhang *et al.*, 1998). BRCA1 has also been shown to transactivate expression of the cyclin-dependent kinase inhibitor p21 in a p53-independent manner (Somasundaram *et al.*, 1997), suggesting that one of the mechanisms by which BRCA1 regulates cell cycle and suppresses growth is through the induction of p21 (Somasundaram *et al.*, 1997).

p53 is a suppressor protein that is altered in numerous human malignancies including colon, lung, breast, ovary and several others (Hollstein *et al.*, 1991). The p53 protein has been implicated in a number of functions, including cell cycle regulation, response to DNA damage, signal transduction, cellular differentiation and activation and repression of transcription (Ko

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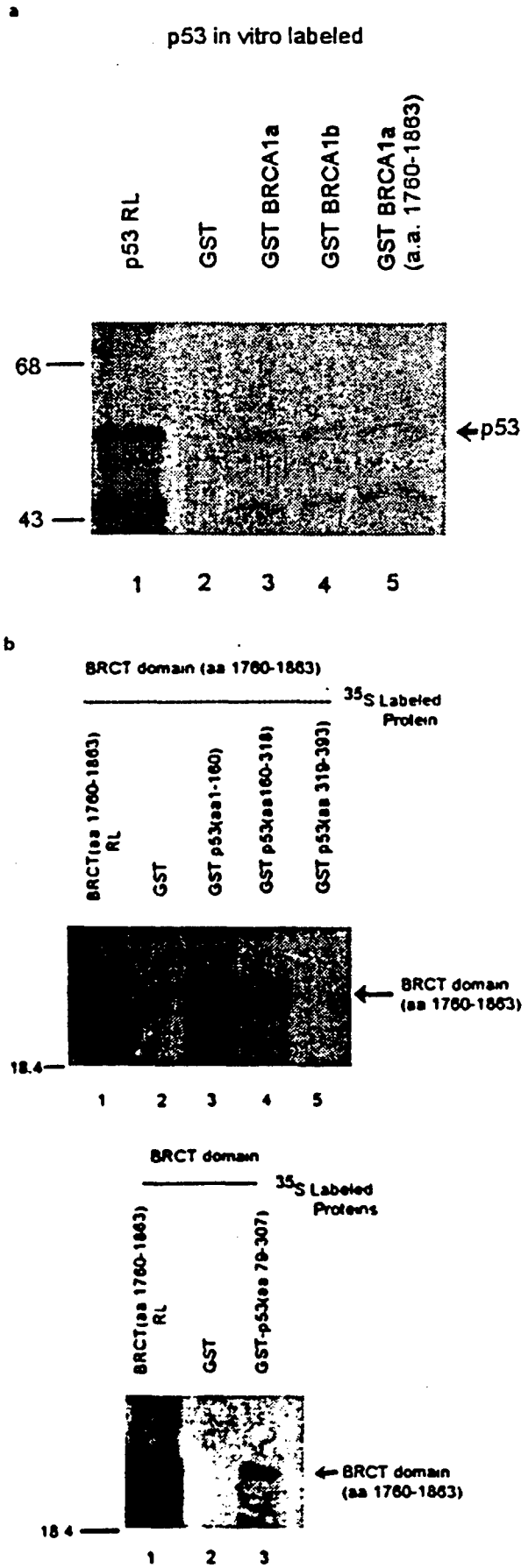

Figure 1 Direct physical association of p53 with BRCA1a, BRCA1b and the second BRCT domain of BRCA1, *in vitro*. (a) GST, GST-BRCA1a, GST-BRCA1b and GST-BRCT domain (amino acids 1760–1863) proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methionine-labeled p53 and subjected to GST-pull down assay as described previously (Wang *et al.*, 1997; Cui *et al.*, 1998b). In lane 1, 1/20th of the *in vitro* translated protein used for binding was loaded directly. The second protein band seen in lane 1 represents polypeptide arising as a result of initiation at the internal methionine codon or premature termination. (b) BRCT domain (amino acids 1760–1863) binds to the central domain of p53 (amino acids 99–307). GST, GST-p53 (amino acids 1–160), GST-p53 (amino acids 160–318), GST-p53 (amino acids 319–393) and GST-p53 (amino acids 99–307) proteins were expressed, purified on beads and incubated with *in vitro* translated ^{35}S-methione-labeled BRCT domain (amino acids 1760–1863) and subjected to GST-pull down assay as described above

and Prives, 1996). The p53 protein contains distinct functional domains: a transcriptional activation domain at the amino terminal (residues ~1–70); a SH3-like domain (residues ~61–94); a central sequence-specific DNA binding domain (residues ~102–392); a nonspecific DNA-binding domain at the carboxy terminal region (residues ~320–393) which contains a tetramerization domain (Stürzbecher *et al.*, 1992); and a basic domain (Ko and Prives, 1996 and references therein).

The presence of a common BRCT domain between BRCA1 and p53 binding protein 53BP1 (Iwabuchi *et al.*, 1994), which is also present in splice variants BRCA1a and BRCA1b (Cui *et al.*, 1998a), led us to investigate whether BRCA1 proteins associate with p53, the universal tumor suppressor, as speculated earlier by others (Bork *et al.*, 1997; Koonin *et al.*, 1996). To determine whether intact BRCA1a/1b and a deletion mutant (containing the second BRCT domain aa 1760–1863) bind with p53, *in vitro* binding was performed with full length wild type human p53 protein. The *in vitro* translated ^{35}S-methionine-labeled p53 protein was passed through GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) and a GST control column as described previously (Wang *et al.*, 1997; Cui *et al.*, 1998b). p53 was found to bind specifically to GST-BRCA1a, GST-BRCA1b, GST-BRCT domain (residues 1760–1863) but not to GST (Figure 1a , lanes 3, 4 and 5). The region of BRCA1/1a/1b extending from aa residues 1760–1863, which also maps to the minimum transactivation domain, was found to be sufficient for interaction with p53. Recently, the N-terminal region of exon 11 (aa 224–500) was shown to interact with the C-terminal domain (aa 300–393) of p53 (Zhang *et al.*, 1998). Interestingly, majority of exon 11 (aa 263–1365) is lost in BRCA1a and BRCA1b (Cui *et al.*, 1998a; Rao, unpublished results) but they still bind to p53. Our results demonstrate for the first time a second p53 interaction domain in the second BRCT domain of BRCA1 protein. To map the region of p53 which binds to the BRCT domain, a series of p53 deletion mutants expressed as GST fusion proteins (generous gifts from Drs Shenk and Wiman) were incubated with *in vitro* translated BRCT domain (aa 1760–1863) protein and subjected to GST-pull down assay. The results show that BRCT domain binds to the central region but not to the C-terminal region (amino acids 319–393) (Figure 1b, top panel lanes 3 and 4, bottom panel lane 3). These results suggest that the BRCT domain of BRCA1 protein binds to the central domain of p53 similar to 53BP1, 53BP2 and SV40 large tumor antigen. This central domain is responsible for site

specific DNA binding and contains mutations that are found commonly in tumors. Since BRCA1a, BRCA1b and the BRCT domain interact *in vitro* with p53, we investigated the effects of BRCA1a/1b and BRCT on p53 independent and p53 dependent transcription. Since both p53 and BRCA1 can transactivate the p21 promoter (El-Deiry *et al.*, 1995; Somasundaram *et al.*, 1997), we studied the effects of BRCA1a and BRCA1b on the p21 promoter. pcDNA-BRCA1a, pcDNA-BRCA1b or pcDNA-BRCT (1760–1863), both alone and in the presence of pSGp53 were co-transfected into COS-7 cells together with a natural genomic p21 promoter CAT vector (a generous gift from Dr Volgelstein) that contains the p53 response element (El-Deiry *et al.*, 1995). Neither BRCA1a, BRCA1b nor BRCT domain (residues 1760–1863) activated the p21 promoter in the absence of exogenous p53 (Figure 2), but all stimulated the p21 promotor in the presence of p53 (Figure 2). These results suggest that BRCA1a and BRCA1b proteins function as co-activators of p53 and the C-terminal second BRCT domain of BRCA1/1a/1b proteins is sufficient for binding to p53 and stimulating its target gene activation. As mentioned earlier, several groups have shown that BRCA1 regulates p21 through both p53-dependent and p53-independent mechanisms (Ouchi *et al.*, 1998; Zhang *et al.*, 1998; Somasundaram *et al.*, 1997). Since BRCA1a/1b lack the N-terminal region of exon 11 which was also shown to interact with p53, we speculate that BRCA1a/p110 and BRCA1b/p100 can regulate p21 transcription only through a p53-dependent mechanism. All these results taken together suggest a role for p53 in the growth arrest, tumor suppression and apoptosis-inducing functions of BRCA1 proteins.

To study these associations in a physiologically relevant environment, cell extracts from human breast cancer cells CAL-51 were immunoprecipitated with anti-BRCA1 polyclonal antibody and as a negative control, normal rabbit IgG. Subsequent Western blot analysis with anti-p53 monoclonal antibody revealed p53 in the anti-BRCA1 immunoprecipitates (Figure 3a, lane 2), and nuclear extract (Figure 3a, lane 3), but not in the control immunoprecipitate (Figure 3a, lane 1). The anti-BRCA1 antibody C-20 detects both BRCA1a/1b proteins by immunoprecipitation and Western blot analysis (Figure 3b). All these results suggest that BRCA1a/1b proteins associate with p53*in vivo*.

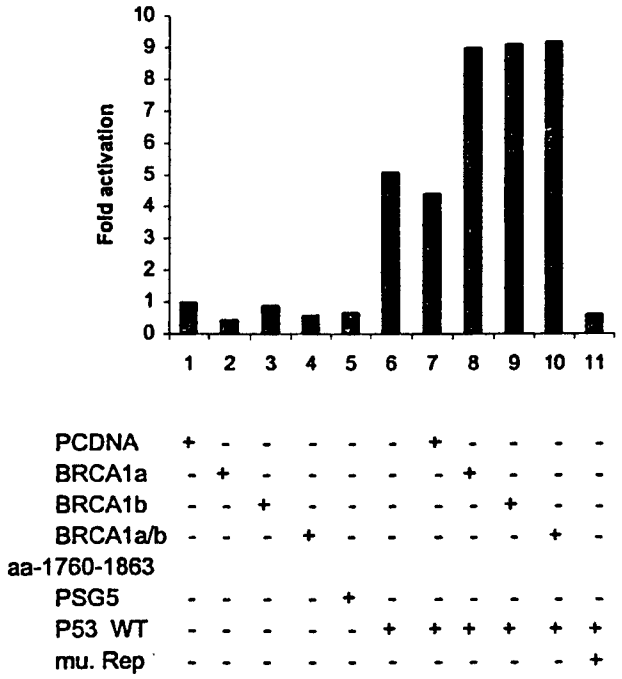

Figure 2 The second BRCT domain of BRCA1/1a/1b is sufficient for p53 dependent transactivation of the p21$^{WAF1/CIP1}$ promoter. COS-7 cells were co-transfected with 1 μg of human p21 promoter CAT 'A' or 'E' construct (El-Deiry *et al.*, 1995) and 1 μg of expression plasmids (pcDNA BRCA1a, pcDNA-BRCA1b, pcDNA-BRCT amino acids 1760–1863 and pSGp53 wild type) as described previously (Cui *et al.*, 1998a,b). Total DNA was kept constant at 20 μg. The CAT activity shown represents fold activity compared with pcDNA vector alone. The activity of the vector is normalized to a value of one. The experiments were repeated at least four times. Mu-Rep represents p21 promoter construct 'E' (El-Deiry *et al.*, 1995) which lacks p53 binding sites. The results of CAT assay were quantitated using a Fuji phosphoimager

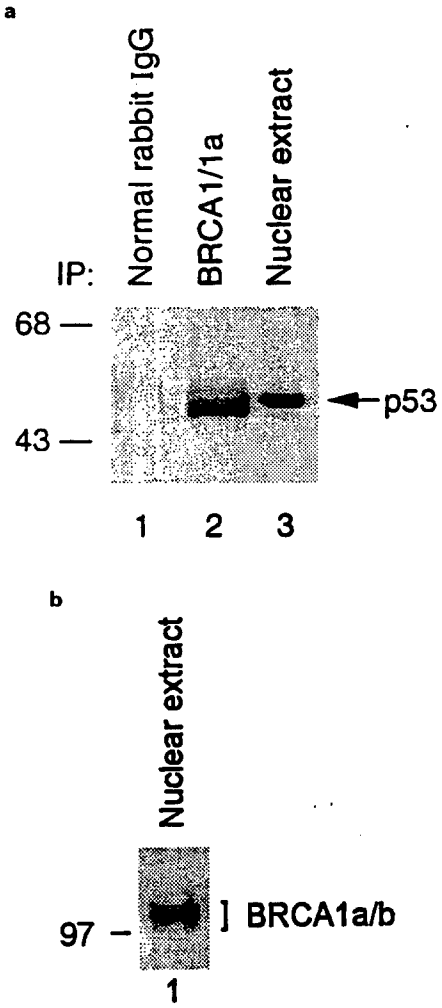

Figure 3 *In vivo* interaction of p53 with BRCA1a/1b proteins. (a) BRCA1 proteins were immunoprecipitated by anti-BRCA1 antibody from cell extracts of human breast cancer cell line CAL51, separated on 10% SDS–PAGE analysis and Western blotted with anti-p53 monoclonal antibody. Lane 1, immunoprecipitation by normal rabbit IgG (negative control), lane 2, immunoprecipitation by anti-BRCA1 antibody, lane 3 A431 nuclear extract (positive control for p53). (b) Western blot analysis of nuclear extract from A431 cells using anti-BRCA1 antibody after immunoprecipitation with anti-BRCA1 antibody was done as described (Cui *et al.*, 1998b). The protein bands shown represent endogenous BRCA1a (p110) and BRCA1b (p100) proteins

BRCA1 associates with RNA polymerase II holoenzyme complex (Scully *et al.*, 1997a) and CBP (CREB-binding protein) is a component of the holoenzyme (Nakajima *et al.*, 1997). Previously, we have found BRCA1a/p110 and BRCA1b/p100 to interact both *in vitro* and *in vivo* with the carboxy-terminal domain of transcription factor CBP (Cui *et al.*, 1998). Since the BRCT domain is sufficient for p53 mediated transactivation of the p21 promoter, we speculated whether the BRCT domain could interact directly with CBP which would bring the RNA polymerase II holoenzyme into play. Our results using GST-pull down assays demonstrate direct physical interaction between GST-CBP2 and *in vitro* translated BRCT domain of BRCA1 (Figure 4a). These data are consistent with the notion that BRCA1 functions as a transcriptional co-activator.

In summary, our results suggest BRCA1a and BRCA1b proteins function as co-activators of p53 tumor suppressor protein similar to BRCA1. This study demonstrates for the first time the presence of a second p53 interaction domain in the carboxy terminal BRCT domain of BRCA1 (Figure 4b), which is sufficient for activation of p53 dependent transactivation of the p21$^{WAF1/CIP1}$ promoter. Previously, based on its homology with p53 binding protein 53BP1, it was speculated that the BRCT domain could bind p53 (Koonin *et al.*, 1996; Bork *et al.*, 1997) and our result

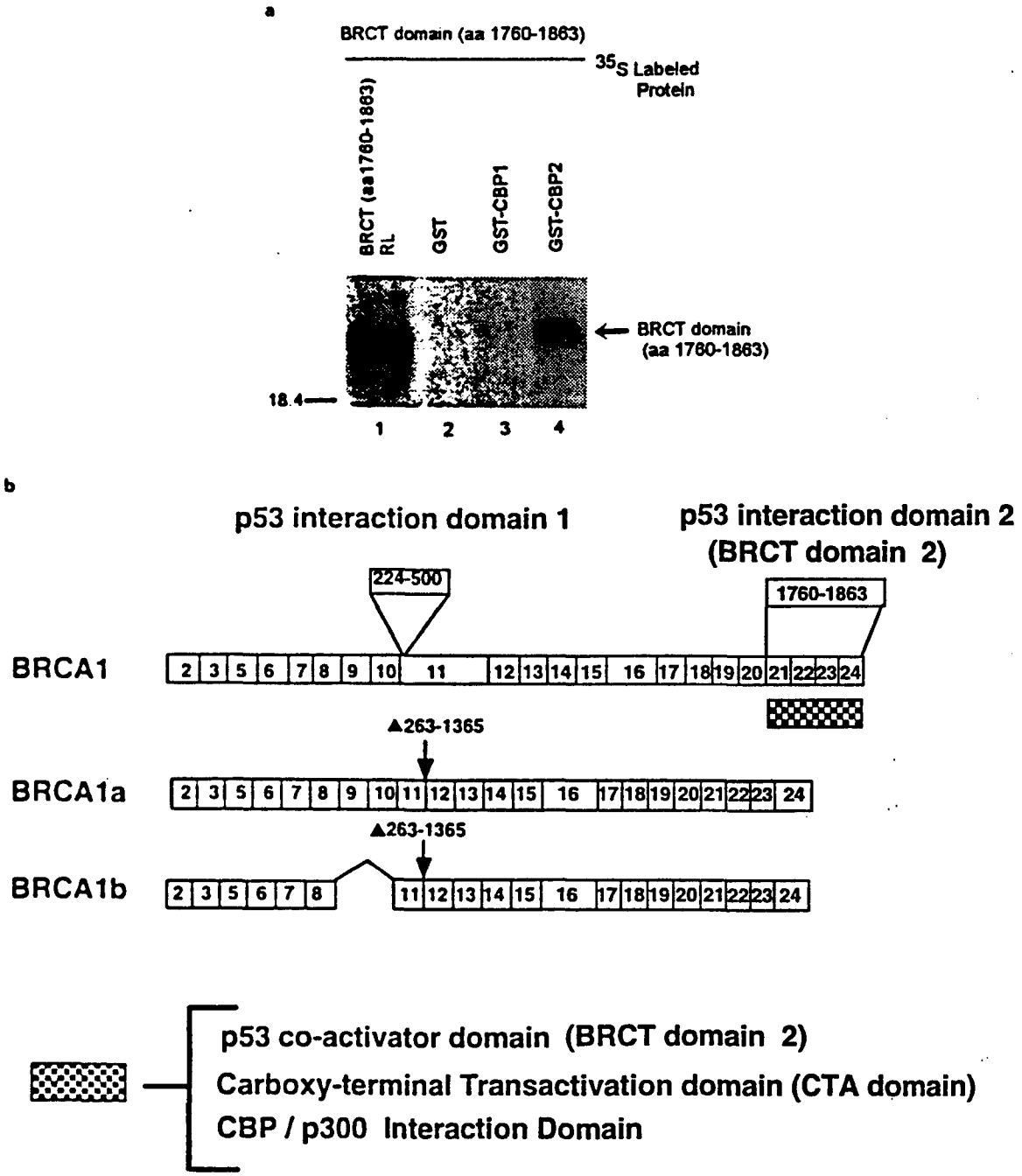

Figure 4 (a) BRCT domain (aa 1760–1863) interacts *in vitro* with CBP2 (aa 1620–1877). GST, GST-CBP1 or GST-CBP2 were incubated with *in vitro* translated ^{35}S-labeled BRCT domain (aa 1760–1863) and subjected to GST-pull down assay as described previously (Cui *et al.*, 1998b) The faint band in lane 3 represents weak binding of BRCT domains to GST-CBP1. (b) Schematic representation of the p53 interaction domains of BRCA1/BRCA1a and BRCA1b proteins

confirms this hypothesis. Since BRCA1a, BRCA1b, and BRCT domain (residues 1760–1863) proteins associate with CBP/p300 co-activator (Cui *et al.*, 1998b and this paper) and the C-terminal truncation, Y1853X, fails to bind to RNA polymerase II holoenzyme (Scully *et al.*, 1997a) nor activate transcription (Somasundaram *et al.*, 1997; Chapman and Verma, 1996; Monteiro *et al.*, 1996; Ouchi *et al.*, 1998; Zhang *et al.*, 1998), we speculate that one of the mechanisms by which BRCA1 proteins function is through recruitment of CBP/p300 associated HAT/FAT activity for acetylation of p53 to specific promoters resulting in transcriptional activation. Our results demonstrate binding of the BRCT domain of BRCA1 (residues 1760–1863) to the central region of p53 (amino acids 79–307) but not to the C-terminal region (amino acids 319–393), which was earlier shown to bind to the N-terminal region of exon 11 of BRCA1 (amino acids 224–500). This central region of p53 contains the sequence specific DNA binding domain which is required for the tumor suppressor function of p53 protein (Ko and Prives, 1996 and references therein). The majority of p53 missense mutations in tumors are present within this central region. The binding of the BRCT domain of BRCA1 proteins to p53 suggests that it may be involved in p53 mediated tumor suppression; apoptosis, and DNA repair. It is possible that the BRCT domain may be involved in determining the specificity of p53 binding to high and low affinity p53 binding sites resulting in either p53-dependent transcriptional activation or repression. It is conceivable that the BRCT domain may activate p53 by inducing a conformational change resulting in increased DNA binding and transactivation. Our findings suggest that the BRCT domain binding to p53 may be mediated by CBP/p300, since it binds to the same region on BRCA1 and this complex may in turn be linked to RNA polymerase II holoenzyme complex. In fact, 87% of mutations that have been reported in familial breast cancer disrupt the BRCT domains by truncation of the protein (Couch and Weber, 1996), while some others disrupt the domains by missense mutations. It remains to be seen what effect these cancer-predisposing mutations have on the p53 binding and co-activator function of BRCA1 proteins. The p53 binding/co-activator property of BRCT domain can be used as an assay for detecting functionally relevant alterations in patients with BRCA1 mutations. It is possible that lack of binding of disrupted BRCA1 proteins to p53 in patients with mutations in the BRCT domain could lead to the development of breast cancer. The binding of the BRCT domain to p53 suggests that other BRCT domain containing proteins involved in cell cycle checkpoint functions responsive to DNA damage may similarly interact with p53. Lastly, although we have shown BRCA1a/1b to activate p53 dependent transcription of the p21 promoter in episome-based assays, the question whether the same holds true for endogenous p21 remains to be investigated.

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Structure and expression of variant BRCA2a lacking the transactivation domain

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Abstract. BRCA1 and BRCA2 are tumor suppressor genes shown to be involved in 90% of familial breast cancers and also known to be involved in ovarian and prostate cancers. Both BRCA1 and BRCA2 gene products are regulated in a cell cycle-dependent manner and have potential transactivation function. Here, we show that BRCA2 undergoes differential splicing giving rise to a novel variant protein BRCA2a, lacking putative transcriptional activation domain. Both BRCA2a and BRCA2 are expressed at high levels in thymus and testis but moderate levels in mammary gland and prostate suggesting that BRCA2a and BRCA2 may have a role in the development and differentiation of these tissues.

Introduction

Germ-line mutations in autosomal dominant susceptibility genes are responsible for up to 10% of all breast cancers (1,2). Mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, could account for up to 90% of familial breast cancers (3-5). Recently BRCA1 and BRCA2 have also been shown to be associated with ovarian and prostate cancers. Interestingly, unlike BRCA1, BRCA2 is associated with male breast cancer (4). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (6,7). Expression of both BRCA1 and BRCA2 was shown to be cell cycle regulated and expressed at high levels in late G1 and S-phase (8,9). Recently exon 3 of BRCA2 was shown to function as a transcriptional activation domain suggesting that BRCA2 may have a functional role in the regulation of transcription (10). Similar transcriptional activation function was also shown to be present in BRCA1 (11-13). Previously we have shown that BRCA1 is trans-ported into the nucleus in the absence of serum and interacts with transcriptional factor E2F, cyclins and cdks suggesting a role for BRCA1 in cell cycle regulation (14). BRCA2 and BRCA1 proteins were shown to interact with Rad 51 suggesting that they may play a role in DNA repair (15-18).

Recently we have shown that both BRCA1 and BRCA2 interact with CBP *in vivo* and *in vitro* suggesting that both these proteins may regulate transcription through CBP (19,20). Because of coordinated expression of BRCA1 and BRCA2 genes and association of these genes in the common breast cancer phenotype, it appears that BRCA1 and BRCA2 may function in a similar pathway. Recently, we have shown that BRCA1 induces apoptosis suggesting a novel function in the regulation of apoptosis of cells (21,22). It remains to be seen whether BRCA2 plays a similar role in apoptosis. Both BRCA1 and BRCA2 proteins may have multicellular functions such as transcriptional activation, DNA repair and regulation of apoptosis.

In this study, we have cloned alternatively spliced BRCA2 cDNA and characterized them by nucleotide sequence analysis. We demonstrate that this differentially spliced BRCA2a transcript has lost transcriptional activation domain as a result of alternative splicing giving rise to BRCA2a with potential dominant negative pathophysiology. Interestingly, BRCA1 was also shown to encode multiple products as a result of alternative splicing (13,14,21,23-25).

Materials and methods

Molecular cloning of BRCA2 and BRCA2a cDNAs. cDNAs were obtained by the reverse transcription of total RNA from BT-474 cells using cDNA kit (Takara). BRCA2 cDNAs were amplified by PCR using appropriate 5' and 3' primers and cloned into a pcDNA3 vector. These cDNAs were characterized by restriction mapping and nucleotide sequence analysis.

RNase protection assay. RNase protection assay was carried using Ribonuclease Protection assay kit (Ambion Inc., Austin TX) as described by the manufacturer. Briefly, the templates were subcloned, linearized and transcribed in 20 µl of *in vitro* transcription mixture containing 5 µl of α-^{32}P-rUTP to obtain radiolabelled probes. These radiolabelled RNA probes were purified by gel electrophoresis. Approximately $5x10^5$ cpm of the probe was mixed with 20 µg of human breast, prostate, testis and thymus RNA (Clontech, Palo Alto, CA) and the

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Key words: BRCA2a, BRCA2, alternative splicing, transcriptional activation, tumor suppressor, HAT activity

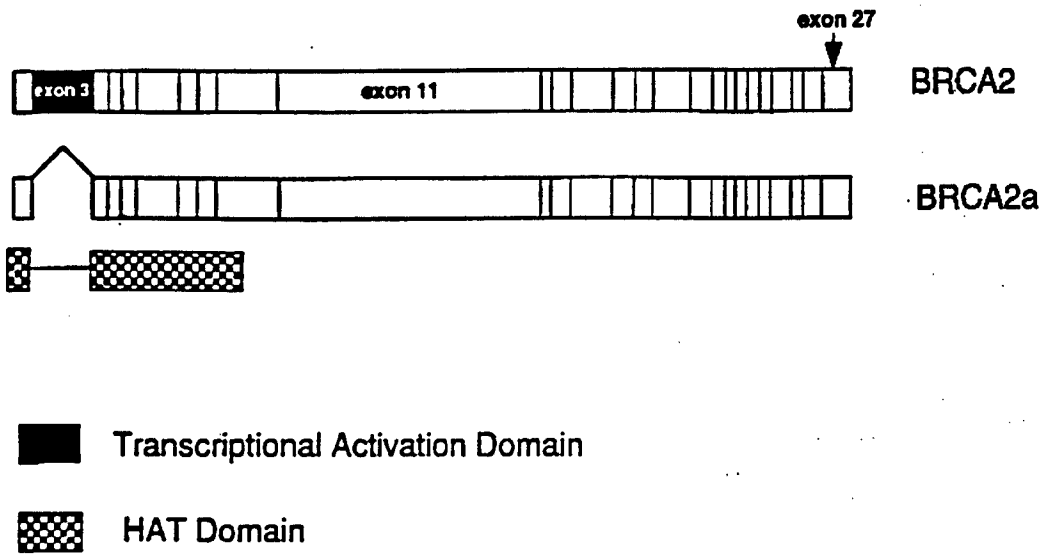

Figure 1. Deletion of BRCA2 transcriptional activation domain in BRCA2a as a result of alternative splicing. Figure is not drawn to scale.

volume of the reaction mixture was adjusted to 30 µl by 1X hybridization buffer. The hybridization mixture was heated to 95°C for 5 min and then incubated at 45°C overnight. RNase digestion was performed for 1 h at 37°C and the reaction was stopped by the addition of RNase inactivation buffer (kit). The protected fragments were extracted by centrifugation. The pellets were suspended in gel loading buffer, heated to 90°C for 3 min. The reaction products were analyzed by polyacrylamide gel electrophoresis using 5% polyacrylamide/8M urea gels. The gel was dried and subjected to autoradiography.

Results and Discussion

In order to understand the function of BRCA2, we have cloned several cDNAs by RT PCR and characterized these cDNAs by nucleotide sequence and restriction map analysis. Our results demonstrate that one of the cDNAs (BRCA2a) showed alternative splicing resulting in the deletion of exon 3 (Fig. 1). Previously this exon was shown to contain a potential transcriptional activation domain, which suggested that BRCA2 may function as a transcriptional factor (10). Similar potential for transcriptional activation was attributed to BRCA1 proteins (11-13). Since BRCA2a has lost transcriptional activation domain, it might compete with native BRCA2 in terms of DNA binding or interaction with other transcriptional factors resulting in dominant negative effect on transcription activation function of BRCA2. Such dominant negative variants are also seen in other transcriptional activators (26). Therefore, BRCA2a may represent a potential dominant negative variant which may regulate the putative transcriptional activation properties of BRCA2 proteins. Alternatively, BRCA2a may have other functions which do not need transactivation function.

We performed RNase protection analysis to study the expression of BRCA2 and BRCA2a in different types of tissues. For this, we have used the 459 nucleotide probe (Fig. 2a). The predicted 313 nucleotide fragment (corresponding to BRCA2a) and the 255 and 58 nucleotide fragments corresponding to BRCA2 were observed in thymus and testis (Fig. 2, lanes 3 and 4). However, moderate to low level of expression was observed in the case of mammary gland and prostate (Fig. 2, lanes 5 and 6). It appears both BRCA2 and BRCA2a are expressed at similar levels in the tissues tested suggesting both forms of BRCA2 may have a functional role in cell growth and differentiation of testis, mammary gland, prostate and thymus.

In summary, we have presented the results supporting that BRCA2 is alternatively spliced, giving rise to a variant BRCA2a protein which lacks transactivation domain. To our knowledge this is the first report demonstrating the presence of variant BRCA2 protein. Since BRCA2a variant lacks transcriptional activation domain, it can potentially interfere with transcriptional activation properties of BRCA2 by competing with BRCA2 for protein-protein interactions and/or DNA binding. Such variant proteins were also seen in the case of other transcriptional factors. It is possible that BRCA2a may regulate the functional properties of BRCA2. Therefore, it becomes important to study the patient DNA samples for mutations outside the coding region (introns, promoters etc.) as they may alter differential splicing pattern of BRCA2 leading to overexpression of BRCA2a. This overexpression of BRCA2a may interfere with normal BRCA2 function and result in cellular transformation. In support of this hypothesis, large deletions that disrupt exon 3 of BRCA2 were observed in patients of breast and ovarian cancers (27).

Our recent results have shown that both BRCA2 and BRCA2a have histone acetyltransferase activity (HAT) (20). These results suggest that domains responsible for HAT activity and the transcriptional activation function of BRCA2 do not overlap (Fig. 1). Since BRCA2 and BRCA2a are HAT proteins, it is possible both BRCA2 and BRCA2a function as transcriptional co-factors. BRCA1 was shown to function as a transcriptional co-factor of p53 (28,29). Recently, we have observed that BRCA2 binds to CBP and function as transcriptional co-factors of p53 (Siddique and Reddy, unpublished results). It is possible that some transcriptional

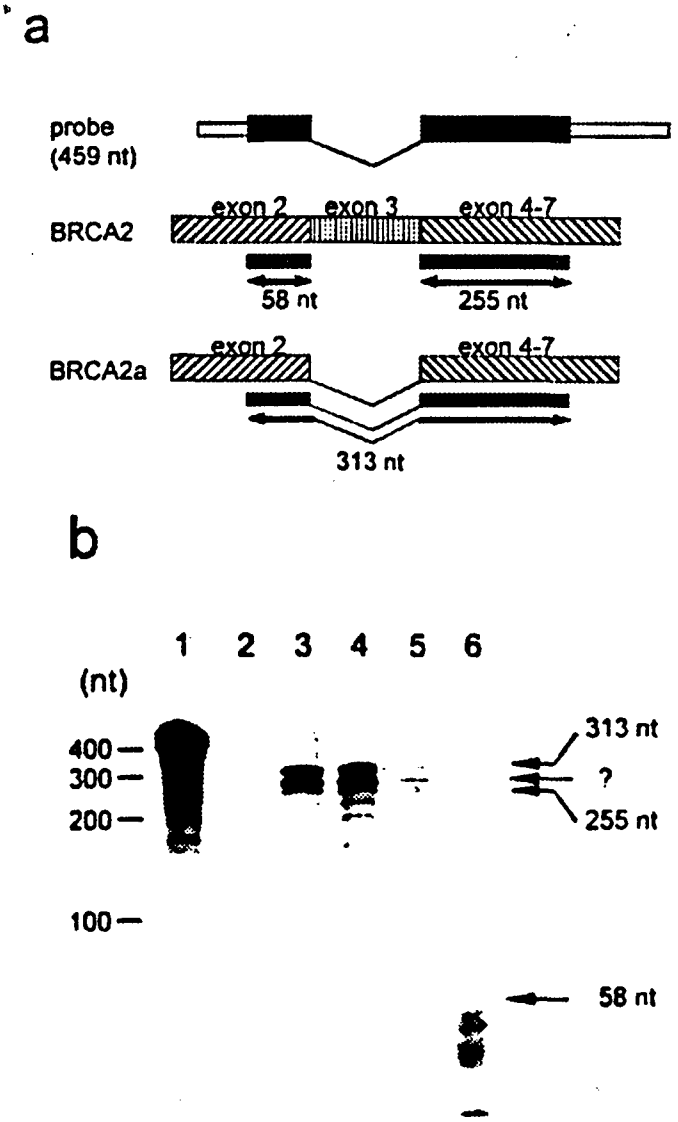

Figure 2. a) Schematic representation of antisense RNA probe used for RNase protection assay. The expected protected fragments from BRCA2 and BRCA2a transcripts are shown. b) Analysis of the expression of BRCA2a in different human tissues by RNase protection assay. This assay was carried out on 20 μg of total RNA using Ambion RPA kit. Lane 1, probe, Lane 2, tRNA, Lane 3, thymus, Lane 4, testis; Lane 5 mammary gland; Lane 6, prostate. Lanes 1-4 are the result of short exposure (1 day) and lanes 5 and 6 are of longer exposure (3 days). Protected 313 nucleotides (corresponding to the alternatively spliced BRCA2a) and 255 and 58 nucleotide fragments (corresponding to the BRCA2) are shown by arrows.

activators may use BRCA2 and BRCA2a as a transcriptional co-factors and utilize their HAT activity for the activation of gene expression. It is also possible that other transcriptional activators may use BRCA2 but not BRCA2a as a transcriptional co-factor because of the absence of the transcriptional activation domain in BRCA2a. Recent studies revealed that BRCA2 interacts with DNA repair protein Rad 51 (15-17). Earlier, we suggested that BRCA2-Rad 51 complex may disrupt nucleosomal structure using HAT activity of BRCA2 and thereby recognize damaged DNA prior to DNA repair process (20). For such a mechanism, there may not be a need for transactivation function. Therefore, BRCA2 and BRCA2a may have distinct functions. Rad 51-BRCA2a (which lacks transactivation domain) complex may play a role in DNA repair whereas BRCA2 may play a role in the regulation of transcription. It remains to be seen whether both isoforms of BRCA2 play a role in this DNA repair phenomenon. Alternatively, BRCA2 proteins may participate in DNA repair and tumor suppression through BRCA2 target genes.

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SHORT REPORT

Induction of apoptosis by Elk-1 and ΔElk-1 proteins

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Elk-1, an ets related gene codes for at least two splice variants Elk-1, which regulates c-*fos* transcription and ΔElk-1, both of which function as transcriptional activators. To investigate the role of Elk-1 and ΔElk-1 proteins in apoptosis; we have developed rat fibroblast cell lines and human breast cancer cell lines expressing Elk-1 and ΔElk-1. The expression of Elk-1 and ΔElk-1 proteins in the Elk-1/ΔElk-1 transfectants were analysed by immunofluorescence, immunohistochemistry, and Western blot analysis. The Elk-1 unlike ΔElk-1 transfectants showed a shortened and flattened morphology compared to the parental cells. We have found that calcium ionophore treatment of Rat-1 Elk-1, MCF-7 Elk-1, Rat-1 ΔElk-1 and MCF-7 ΔElk-1 transfectants resulted in programmed cell death. These results indicate that constitutive expression of Elk-1 and ΔElk-1 proteins triggers apoptosis in Rat-1 fibroblasts and breast cancer cells when treated with calcium ionophore.

Keywords: Elk-1; ΔElk-1; apoptosis; calcium ionophore; breast cancer; MCF-7; Rat-1

The Elk-1 gene belongs to the ets family of ternary complex factors (TCFs), i.e. Elk-1, SAP1, and NET/ERP/SAP2/Elk-3 (Rao *et al.*, 1989; Hipskind *et al.*, 1991; Giovane *et al.*, 1994; Lopez *et al.*, 1994; Dalton and Treisman, 1992; Price *et al.*, 1995; Nozaki *et al.*, 1996). The Elk-1 gene codes for at least two alternately spliced products Elk-1 (Rao *et al.*, 1989) and ΔElk-1 (Rao and Reddy, 1993) which function as transcriptional activators (Rao and Reddy, 1992; Bhattacharya *et al.*, 1993), are substrates for MAP kinases (Rao and Reddy, 1993; Marias *et al.*, 1993; Hill *et al.*, 1993) and JNK protein kinases (Gupta *et al.*, 1996; Whitmarsh *et al.*, 1995). As mentioned earlier, the Elk-1 protein is a TCF which in association with serum response factor (SRF) forms a ternary complex on the serum response element (SRE) of the c-*fos* promoter and regulates c-*fos* transcription (Hipskind *et al.*, 1991). The TCF's which includes Elk-1 have three domains with similar sequences and functions. The ets domain mediates DNA binding, the SRF interaction domain interacts with SRF to form a ternary complex with the c-*fos* SRE and the C-terminal domain activates transcription upon phosphorylation by MAP kinases (Rao *et al.*, 1989; Rao and Reddy, 1992; Dalton and Treisman, 1992; Janknecht *et al.*, 1993, 1994; Marias *et al.*, 1993; Giovane *et al.*, 1994; Hipskind *et al.*, 1994; Kortenjann *et al.*, 1994; Lopez *et al.*, 1994; Hill *et al.*, 1995; Price *et al.*, 1995; Whitmarsh *et al.*, 1995) and JNK kinases (Gupta *et al.*, 1996). Thus Elk-1 represents a key link between signal transduction and induction of gene transcription.

The Gag-Myb-Ets fusion protein, identified in the avian acute leukemia virus E26 was shown to inhibit apoptosis and induce erythroid differentiation in hematopoietic cells (Athanasiou *et al.*, 1996). Similarly the Ets-1 proto-oncogene was shown to be required for the normal survival and activation of B and T cells while an Ets-1 splice variant was shown to induce apoptosis in human colon cancer cells indicating a role in apoptosis (Bories *et al.*, 1995; Muthusamy *et al.*, 1995; Huang *et al.*, 1997). Recently, erg and fli-1 proteins were shown to inhibit apoptosis (Yi *et al.*, 1997). Mitogen activated/extracellular response kinase kinase kinase (MEKK), a serine-threonine kinase that regulates phosphorylation and activation of mitogen-activated protein kinases (MAPK) was shown to induce cell death (Johnson *et al.*, 1996). Inducible expression of activated MEKK stimulated the transactivation of c-Myc and Elk-1 (Johnson *et al.*, 1996). To date, molecules involved in signaling apoptosis include ceramide (Jimenez *et al.*, 1995), Rho, Ras, c-Myc, p53, E1A (Canman and Kastan, 1995), c-Jun (Evan *et al.*, 1992), Fas (Wang *et al.*, 1994), proteins associated with the TNFα receptor (Chinnaiyan *et al.*, 1995), BRCA1 (Shao *et al.*, 1996), Fos (Preston *et al.*, 1996), E2F-1 (Shan *et al.*, 1996), c-Myc and c-Jun transcription factors which are regulated by MAPK phosphorylation also induce apoptosis (Canman and Kastan, 1995). Since Elk-1 protein regulates c-Fos oncogene and is a target for MAPK and JNK both of which are activated by MEKK, we speculated whether it could play a similar role in inducing an apoptotic response. In this study, we have developed rat fibroblast cell lines and human breast cancer cell lines expressing Elk-1 and ΔElk-1 proteins. Our results suggest that constitutive expression of Elk-1 and ΔElk-1 induce apoptosis in both Rat-1 and MCF-7 cells.

In an attempt to study the function of Elk-1 and ΔElk-1 proteins in the regulation of apoptosis, we have transfected Rat-1 fibroblasts and MCF-7 breast cancer cells with pcDNA expression vector pcDNA expression vectors containing human Elk-1 and

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ΔElk-1 cDNA and obtained stable G418 resistant cell lines expressing Elk-1 and ΔElk-1 proteins. The morphology of the Elk-1 transfectants were different from that of the parental Rat-1 fibroblast and MCF-7 cells. The Elk-1 transfectants were slow growing and appeared to be shorter and flatter compared to the parental Rat-1/MCF-7 cells. The morphology of the ΔElk-1 transfectants were similar to that of the parental cells. We analysed the expression of Elk-1 and ΔElk-1 proteins in Elk-1 and ΔElk-1 transfectants by indirect immunofluorescence analysis, immunoperoxidase staining (Figure 1) and Western blot analysis (Figure 2) using Elk-1 polyclonal antibody as described previously (Shao *et al.*, 1996; Wang *et al.*, 1997). The nuclear and cytoplasmic staining was brighter and stronger in both Elk-1 and ΔElk-1 transfected cells compared to parental Rat-1 fibroblast cells suggesting higher levels of expression of Elk-1 and ΔElk-1 proteins in transfected cells (Figure 1). Western blot analysis revealed a significant increase in the levels of expression of Elk-1 and ΔElk-1 proteins when compared to the parental Rat-1 cells (Figure 2). Similarly, we have also observed significant increase in the expression of Elk-1 and ΔElk-1 proteins in MCF-7 Elk-1 and MCF-7 ΔElk-1 transfectants by immunoperoxidase staining and Western blot analysis (data not given). Calcium ionophore A23187 is known to induce apoptosis in thymocytes and neurons and previously, we have shown A23187 to induce apoptosis in BRCA1a transfected NIH3T3 and MCF-7 cells (Cohen *et al.*, 1984; Joseph *et al.*, 1993; Shao *et al.*, 1996). This led us to examine apoptosis in Rat-1 Elk-1, MCF-7 Elk-1, Rat-1 ΔElk-1 and MCF-7 ΔElk-1 transfectants after A23187 treatment. Rat-1, Rat-1 Elk-1, Rat-1 ΔElk-1 and MCF-7, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells were treated with calcium ionophore A23187 for 24–48 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method. Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M phase of the cell cycle were evaluated using EPICS profile analyser. The Elk-1 and ΔElk-1 transfected cells showed accelerated rates of apoptosis (Ap value 49% for Rat-1 Elk-1, 66% for MCF-7 Elk-1, 42% for Rat-1 ΔElk-1 and 28% for MCF-7 ΔElk-1 cells) in the presence of calcium ionophore (Figure 3a and b)

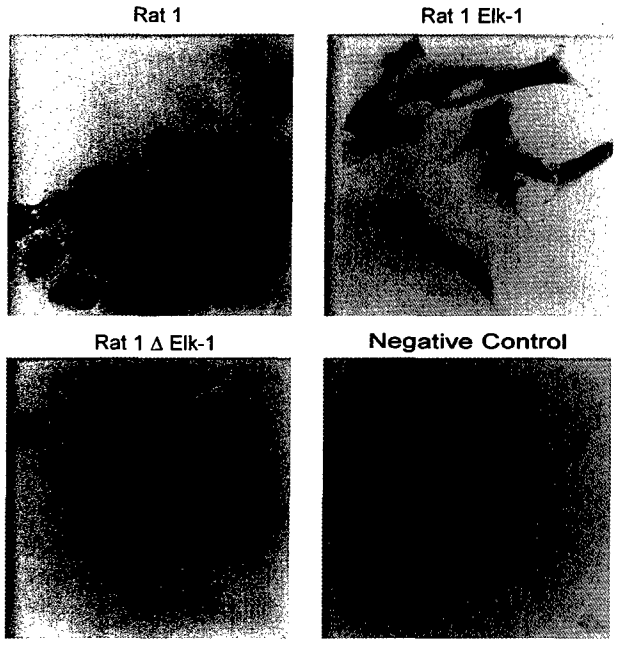


Figure 1 Detection of Elk-1 and ΔElk-1 proteins in Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells by immunohistochemistry. Full length Elk-1 (Rao *et al.*, 1989) and ΔElk-1 (Rao and Reddy, 1993) cDNA's were subcloned into pcDNA expression vectors. Purified DNA (20 μg) of pcDNA expression vector or vector containing Elk-1/ΔElk-1 cDNA's were transfected into Rat-1 and MCF-1 cells by calcium phosphate precipitation method using the Invitrogen kit as described previously (Rao *et al.*, 1996). Rat-1, Elk-1 and ΔElk-1 transfectant cells were cultured in chamber slides and processed for immunohistochemistry using an Elk-1 carboxyterminal peptide antibody as described previously (Rao *et al.*, 1996; Shao *et al.*, 1996)

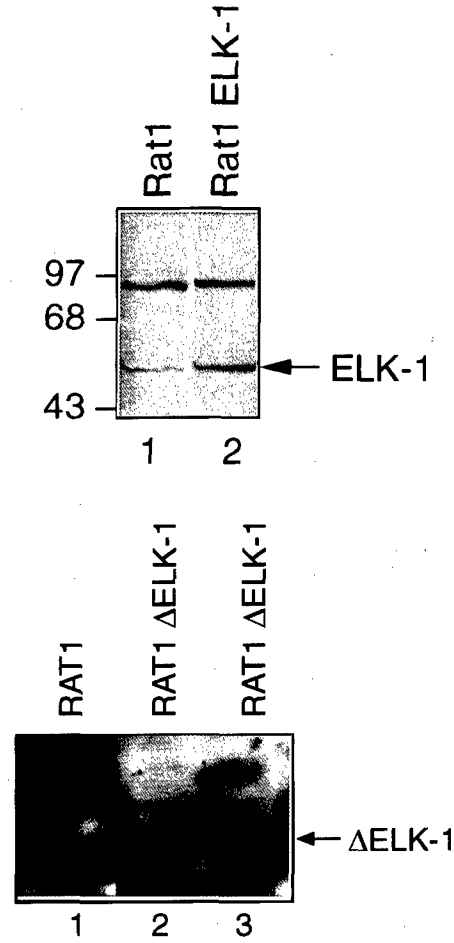


Figure 2 Western blot analysis of Rat-1 cells stably transfected with Elk-1 and ΔElk-1 using an anti-C-terminal Elk-1 antibody. Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells were harvested by washing in PBS and treating with trypsin. For preparing total cell extracts the cells were lysed in RIPA buffer and subjected to Western blot analysis as described previously (Rao *et al.*, 1996). For Western blotting analysis ~50 μg of cell extract in SDS sample buffer were loaded on a 10% SDS–PAGE in Bio-Rad mini-protein II cell as described previously (Rao *et al.*, 1996). After electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membrane (Amersham). Immunodetection of Elk-1 protein was performed with a polyclonal anti-Elk-1 peptide antibody (Santa Cruz Biotechnology, I-20) diluted 1:100 using ECL Western exposure chemiluminescent detection system from Amersham

whereas the control Rat-1 fibroblasts and MCF-7 cells showed lower levels of apoptosis under identical conditions (Ap value 16% and 5% respectively).

Measurement of apoptosis through the sub G1 peak in the DNA histogram gives no distribution between viable and dead cells since all the cells are fixed. We, therefore, studied the viability of Rat-1 cells, Rat-1 Elk-1, Rat-1 ΔElk-1, MCF-7 cells, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells cultured in the presence of calcium ionophore by MTT metabolic assay. In this assay, viable cells will cleave MTT to produce formazan using mitochondrial enzyme succinate tetrazolium reductate and the quantity of formazan dye produced correlates directly to the number of viable cells. As shown in Figure 3c, only 35% of Rat-1 Elk-1 cells and 33% of Rat-1 ΔElk-1 cells remained viable upon exposure to calcium ionophore for 24 h, compared to nearly 80% of control Rat-1 cells. Similarly, (Figure 3d) only 21% of MCF-7 Elk-1 and 24% of MCF-7 ΔElk-1 cells survived upon exposure to calcium ionophore for 24 h, compared to nearly 40% of control MCF-7 cells. These results suggest that both Elk-1 and ΔElk-1 proteins induce death in Rat-1 and MCF-7 cells.

Elk-1 and ΔElk-1 transfected Rat-1 cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hoescht 33258. The nuclei of Rat-1 Elk-1 and Rat-1 ΔElk-1 cells showed strong chromatin condensation and nuclear degradation into small, spherical nuclear particles of condensed chromatin characteristic of apoptosis, whereas the parental Rat-1 cells did not show any significant change in the staining pattern (Figure 3e).

The induction of apoptosis in the Elk-1 and ΔElk-1 transfectants upon treatment with calcium ionophore was further confirmed by analysis of DNA fragmentation. The DNA of Elk-1 and ΔElk-1 cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder typical of apoptosis whereas the parental Rat-1/MCF-7 cells showed no significant DNA degradation (Figure 3f and g). These results suggested that calcium ionophore induces apoptosis more readily in Rat-1 Elk-1, MCF-7 Elk-1, Rat-1 ΔElk-1, MCF-7 ΔElk-1 cells than control Rat-1/MCF-7 cells and overexpression of Elk-1 and ΔElk-1 induces apoptosis in breast cancer cells. All the above results shown for a single clone of Rat-1 Elk-1 or Rat-1 ΔElk-1 or MCF-7 Elk-1 or MCF-7 ΔElk-1 cells have been reproducibly obtained with several other independent clonal isolates of Rat-1/MCF-7-Elk-1 and Rat-1/MCF-7ΔElk-1 cells. From our data it appears that Elk-1 is more effective in causing cell death than ΔElk-1 when cells are exposed to A23187. These differences in the apoptotic potential of Elk-1 and ΔElk-1 proteins does not appear to be due to differences in the levels of expression of these proteins but may be due to the inherent differences in their physiological functions, since Elk-1 is a ternary complex factor and c-*fos* regulator unlike ΔElk-1 protein.

c-*fos* transcription is induced rapidly by a wide array of extracellular stimuli, including growth factors, cytokines, neurotransmitters, ion fluxes, phorbol esters and UV irradiation. Growth-factors induce c-*fos* transcription by stimulating phosphorylation of transcriptional factor TCF/Elk-1 by MAPKs while UV irradiation and MEKK1 activation strongly activate two other MAPKs JNK1 and JNK2, which also stimulate Elk-1 transcriptional activity and phosphorylation. In short UV irradiation and MEKK1 activation stimulates Elk-1 activity through JNK activation and growth factors induce Elk-1 phosphorylation, c-*fos* transcription through ERK activation. Recently, MEKK which phosphorylates and activates MAPK, JNK and SAPK's was shown to regulate signal transduction pathways that contribute to the apoptotic response (Johnson *et al.*, 1996). Apoptosis is a highly regulated process of cell death that is characterized by cell shrinkage, chromatin condensation and cellular DNA fragmentation (Searle *et al.*, 1982). The process of programmed cell death appears to be regulated by proteins that also function in proliferation and differentiation. For example, both c-Myc and c-*fos* which are immediate early gene products have been shown to induce apoptosis (Canman and Kastan, 1995; Preston *et al.*, 1996). The gag-Myb-Ets fusion protein, c-ets-1 proto-oncogene, and two ets superfamily members namely erg and fli-1 have been shown to inhibit apoptosis whereas ets-1 splice variant has been shown to induce apoptosis indicating a role for the ets family of genes in apoptosis (Athanasiou *et al.*, 1996; Bories *et al.*, 1995; Muthusamy *et al.*, 1995; Yi *et al.*, 1997; Huang *et al.*, 1997). Our findings on the Elk-1 proteins is consistent with a role for the ets related genes in cell death.

In summary our results demonstrate for the first time, a role for Elk-1 and ΔElk-1 proteins in mediating a cell death response characteristic of apoptosis. Thus Elk-1 and ΔElk-1 proteins induce apoptosis similar to Rho, Ras, c-Myc, c-*jun*, Fas, BRCA1, Fos, p53, E1A, E2F-1, etc (Canman and Kastan, 1995; Evan *et al.*, 1992; Wang *et al.*, 1994; Shao *et al.*, 1996; Preston *et al.*, 1996; Shan *et al.*, 1996). The precise mechanism by which Elk-1 induces cell death remains to be investigated. Since Elk-1 functions as a transcriptional regulator, it may be either activating death inducing genes like *fos* (Preston *et al.*, 1996), Bad, Bax, Bak, Bcl-xL, etc. or repressing death inhibiting genes like Bcl-2 (Oltvai and Korsmeyer, 1994; Reed, 1994). Bcl-xL, Mcl-1, Al, Bag-1, etc. leading to apoptosis.

Alternatively, Elk-1 may activate apoptosis inducing proteins or target apoptosis inhibiting proteins through direct protein–protein interactions. Elk-1 could behave similar to the c-*myc* oncogene which is implicated both in the control of normal cellular proliferation and apoptosis (Evan *et al.*, 1992). Recent evidence suggests that decrease in normal cell death is a characteristic of breast cancers (Thompson, 1995). Future efforts will be directed towards delineating the mechanisms (such as induction of Bcl-2 family members or caspases or JNK pathways) by which Elk-1 and ΔElk-1 proteins induce apoptosis.

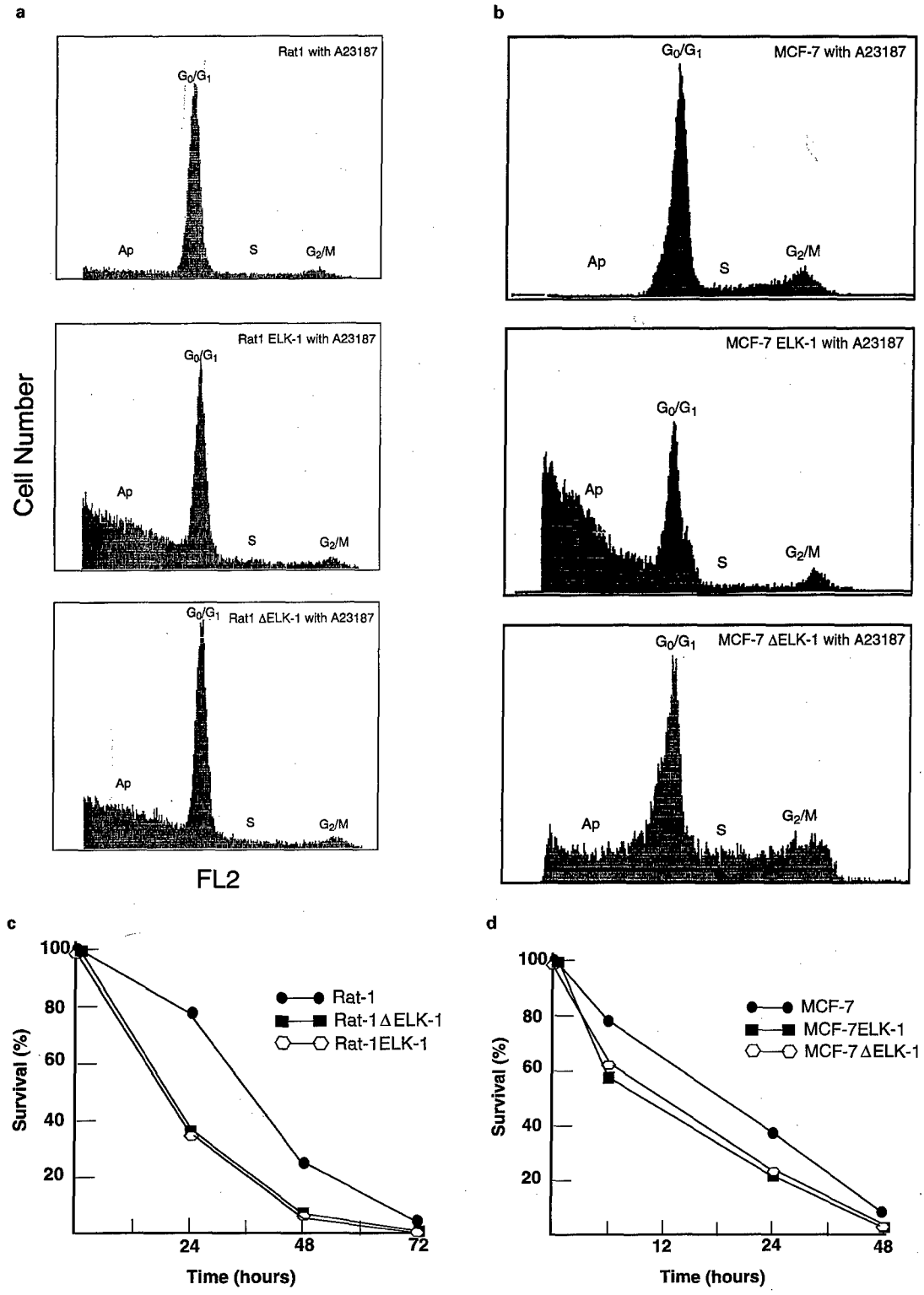
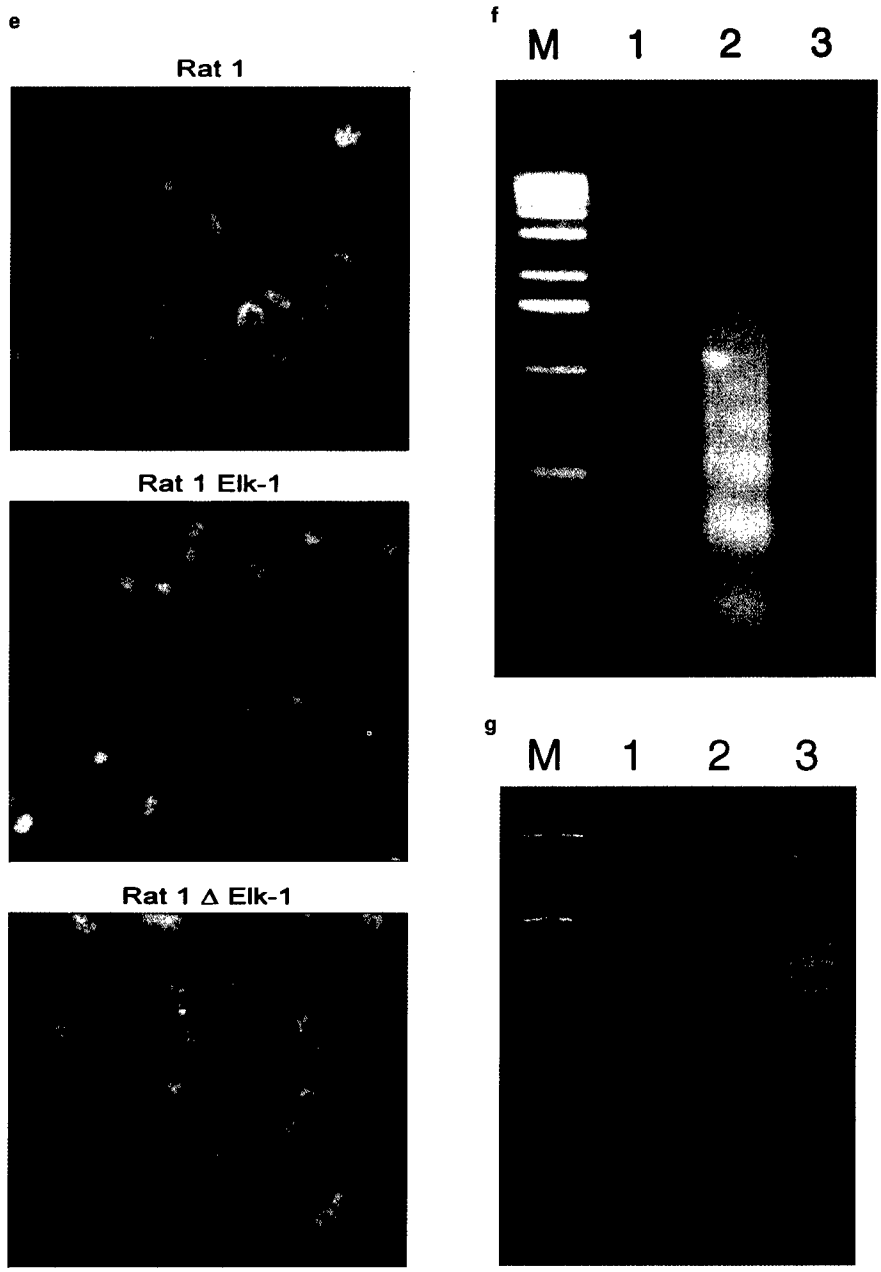


Figure 3 Elk-1 and ΔElk-1 proteins induce apoptosis in Rat-1 and MCF-7 cells after calcium ionophore treatment. (**a**) Flow cytometric analysis of Rat-1 cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 48 h. (**b**) Flow cytometric analysis of MCF-7 cells triggered to undergo apoptosis by A23187 treatment for 24 h. Subconfluent Rat-1, Rat-1 Elk-1, Rat-1 ΔElk-1, MCF-7, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells were incubated in their respective media with or without 20 μM calcium ionophore A23187. After 24–48 h, both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at −18°C overnight. Cells were stained with propidium iodide (20 μg/ml) and incubated with 20 μg/ml of RNAase A at 37°C for 30 min. Samples were analysed using a EPICs profile analyser. Histograms showing the total DNA content as FL2 *vs* Cell numbers are shown. (**c**) Time course of A23187 induced cell death in Rat-1, Rat-1 Elk-1, and Rat-1 ΔElk-1 cells. Percentage of surviving cells were measured by modified MTT assay. 5×10^4 cells/well were seeded into 24 well culture dishes and incubated at 37°C, 5% CO_2 for 24 h. The medium was removed and the cells were cultured in complete media containing 20 μM calcium ionophore A23187 (Sigma). At different times, 0.1 ml of MTT (5.0 mg/ml in PBS) solution was added into each well and incubated at 37°C, 5% CO_2 for another 3 h. The medium was discarded, 0.5 ml of 0.04 HCl-isopropranol was added into each well and kept at room temperature for 20 min. Then 0.5 ml of PBS was added and quantitative by measurement of



absorbance at 570 nm. The difference of absorbance before and after the treatment with A23187 represent the number of surviving cells. **(d)** Time course of A23187 induced cell death in MCF-7, MCF-7 Elk-1 and MCF-7 ΔElk-1 cells. The percentage of surviving cells was measured by modified MTT assay. **(e)** Chromatin condensation shown morphologically by Hoescht staining of Rat-1 Elk-1 and Rat-1 ΔElk-1 cells induced to undergo cell death by calcium ionophore for 48 h. Rat-1, Rat-1 Elk-1 cells and Rat-1 ΔElk-1, cells treated with or without 20 μM calcium ionophore for 24–48 h were cultured on glass cover slips and fixed in methanol: glacial acetic acid (3:1) at −18°C for 30 min. The cells were washed in PBS and stained with 8 μg/ml Hoescht 33258 for 5 min in dark. The cover slips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope. **(f)** DNA fragmentation induced by Elk-1 and ΔElk-1 overexpression in Rat-1 cells. 1, Rat-1 cells treated with calcium ionophore; 2, Rat-1 Elk-1 cells treated with calcium ionophore; 3, Rat-1 ΔElk-1 cells treated with calcium ionophore. **(g)** DNA fragmentation induced by Elk-1 and ΔElk-1 overexpression in MCF-7 cells. Lane 1, MCF-7 cells treated with calcium ionophore; 2, MCF-7 ΔElk-1 cells treated with calcium ionophore; 3, MCF-7 Elk-1 cells treated with calcium ionophore. This assay was modified from the one previously described (Kondo *et al.*, 1994). Briefly, subconfluent Rat-1, Rat-1 Elk-1 and Rat-1 ΔElk-1 cells were treated with calcium ionophore A23187. After 48 h, both adherent and nonadherent cells were pooled (1×10^6) and washed once with PBS. The cell pellets were lysed in 1.0 ml of a buffer containing 10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysates were centrifuged (13 000 *g*) for 10 min at 4°C in an Eppendorf microfuge. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol: chloroform: isoamylalcohol, then with chloroform isoamylalcohol (24:1). The aqueous phase was made to 300 mM sodium chloride and the nucleic acids were precipitated with two volumes of ethanol. The pellet was rinsed with 70% ethanol, air dried and dissolved in 40 μl of TE buffer (pH 8.0). Following digestion of RNA with RNAase A (100 μl/ml, at 37°C for 30 min), the samples were electrophoresed on 2% agarose gel. DNA was then visualized after ethidium bromide staining. DNA fragmentation analysis of MCF-7, MCF-7 Elk-1 and MCF-7 ΔElk-1 were done as described above except for the treatment of A23187 (20 μM) for 24 h

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